Soil Microbial Community Function and Structure as Assessment Criteria for the Rehabilitation of Coal Discard Sites in South Africa

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This work is dedicated to my parents, Francois and Sunette. I have deepest gratitude for their patience, encouragement and loving support throughout my entire University career and in everything I endeavour. Thank you for this wonderful opportunity. I love you.

"Never regard study as a duty, but as the enviable opportunity to learn, to know the liberating influence of beauty in the realm of the spirit, for your own personal joy and to the profit of the community to which your work later belongs."

– Albert Einstein

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DECLARATION

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The study represents original work undertaken by the author and has not been previously submitted for degree purposes to any other university. Appropriate acknowledgements in the text have been made where the use of work conducted by other researchers have been included.

Sarina Claassens

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Language and style used in this dissertation are in accordance with the requirements of the journal *Soil Biology and Biochemistry.*

This dissertation represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between the chapters has been unavoidable.

SUMMARY

Mining activities cause severe disturbance to the soil environment in terms of soil quality and productivity and are of serious concern worldwide. Under South African legislation, developers are required to ecologically rehabilitate damaged environments. The application of agronomic approaches for the rehabilitation of coal discard sites has failed dismally in the arid areas of southern Africa. It is obvious that compliance with mitigation and rehabilitation requirements cannot be enforced without a thorough understanding of the ecological principles that ensure ecological stability and subsequent sustainability of soil ecosystems. Soil microorganisms are crucial role-players in the processes that make energy and nutrients available for recycling in the soil ecosystem. Poor management practices and other negative impacts on soil ecosystems affect both the physical and chemical properties of soil, as well as the functional and structural properties of soil microbial communities. Disturbances of soil ecosystems that impact on the normal functioning of microbial communities are potentially detrimental to soil formation, energy transfers, nutrient cycling, plant reestablishment and long-term stability. In this regard, an extensive overview of soil properties and processes indicated that the use of microbiological and biochemical soil properties, such as microbial biomass, enzymatic activity and the analysis of microbial community structure by the quantification of specific signature lipid biomarkers are useful as indicators of soil ecological stress or restoration properties because they are more responsive to small changes than physical and chemical characteristics. In this study, the relationship between the physical and chemical characteristics and different biological indicators of soil quality in the topsoil covers of seven coal discard sites under rehabilitation in South Africa, as well as three reference sites was investigated. Through the assimilation of basic quantitative data and the assessment of certain physical, chemical and biological properties of the topsoil covers obtained from the various coal discard sites as well as the reference sites, the relative success or progress of rehabilitation and the possible correlation between the biological indicators of soil quality and the establishment of self-sustaining vegetation covers was determined. Results from soil physical and chemical analyses and percentage vegetation cover were correlated with the results obtained for the

functional and structural diversity of microbial communities at the various sites. All results were investigated through statistical and multivariate analysis and the most prominent physical and chemical parameters that influence the biological and biochemical properties of the soil and possibly the establishment of self-sustainable vegetation cover on these mine-tailing sites were identified. Results obtained from this study indicated no significant difference ($p>0.05$) between the various discard sites based on conventional microbiological enumeration techniques. However, significant differences ($p<0.05$) could be observed between the three reference sites. All enzymatic activities assayed for the rehabilitation sites, with the exception of urease and alkaline phosphatase displayed a strong, positive association with the organic carbon content (%C). Ammonium concentration had a weak association with all the enzymes studied and pH only showed a negative association with acid phosphatase activity. A positive association was observed between the viable microbial biomass, vegetation cover and the organic carbon content, ammonium, nitrate and phosphorus concentrations of the soil. The various rehabilitation and reference sites could be differentiated based on the microbial community structure as determined by phospholipid fatty acid (PLFA) analysis. It is hypothesised that the microbial community structure of the Hendrina site is not sustainable when classified along an r-K gradient and that the high percentage of vegetation cover and high levels of estimated viable microbial biomass are an artificial reflection of the current management practices being employed at this site. Results obtained during this study, suggest that an absence or low percentage of vegetation cover and associated lower organic matter content of the soil have a significant negative impact on soil biochemical properties (enzymatic activity) as well as microbial population size. Furthermore, prevailing environmental physico-chemical and management characteristics significantly influences the vegetation cover and subsequently the microbial community structure. The results indicate that the microbial ecosystems in the coal discard sites could become more stable and ecologically self-regulating, provided effective management to enhance the organic carbon content of the soil. This could enhance nutrient cycling, resulting in changes of soil structure and eventually an improved soil quality which could facilitate the establishment of self-sustaining vegetation cover. Results obtained during this study suggest that a polyphasic assessment of physical and chemical properties; microbial activities by enzymatic analysis;

the characterisation of microbial community structure by analysis of phospholipid fatty acids; and the multifactorial analysis of the data obtained can be used as complementary assessment criteria for the evaluation of the trend of rehabilitation of mine tailings and discard sites. Strategic management criteria are recommended based on the soil quality/environmental sustainability indices to facilitate the establishment of self-sustainable vegetation covers. The contribution of this research to soil ecology is significant with regards to the intensive investigation and explanation of characteristics and processes that drive ecological rehabilitation and determine the quality of the soil environment. The multidisciplinary approach that is proposed could, furthermore, assist in the successful rehabilitation and establishment of self-sustaining vegetation covers at industrially disturbed areas, as well as assist in improving degraded soil quality associated with both intensive and informal agriculture. Additionally, this approach could negate the negative social and environmental impacts frequently associated with these activities.

Key terms: *Coal discard; Enzymatic activity; Microbial activity; Microbial community structure; Phospholipid fatty acids; Rehabilitation; Soil quality*

OPSOMMING

Mynwerksaamhede veroorsaak geweldige versteuring in die grondomgewing in terme van grondkwaliteit en –produktiwiteit en is wêreldwyd 'n ernstige rede tot kommer. Suid-Afrikaanse wetgewing vereis dat ontwikkelaars versteurde omgewings ekologies rehabiliteer. Die aanwending van landboukundige benaderings vir die rehabilitasie van steenkoolafvalterreine het grootliks misluk, veral in die droë dele van suidelike Afrika. Dit is duidelik dat mitigasie en rehabilitasie vereistes nie toegepas kan word sonder 'n deeglike begrip van die ekologiese beginsels wat ekologiese stabiliteit en gevolglike volhoubaarheid van grondekosisteme moontlik maak nie. Grondmikroörganismes is van kardinale belang tydens die prosesse van energie- en nutriëntvrystelling vir hersirkulering in die grondekosisteem. Swak bestuurspraktyke en ander negatiewe impakte op grondekosisteme affekteer beide die fisiese en chemiese eienskappe van grond, asook die funksionele en strukturele eienskappe van die grondmikrobiese gemeenskappe. Versteurings van grondekosisteme wat op die normale funksionering van mikrobiese gemeenskappe impakteer, is potensiëel skadelik vir grondvorming, energie-oordrag, sirkulering van nutriënte, planthervestiging en langtermyn stabiliteit. In hierdie verband, het 'n uitgebreide oorsig oor grondeienskappe en –prosesse aangedui dat die gebruik van mikrobiologiese en biochemiese grondeienskappe, soos mikrobiese biomassa, ensiemaktiwiteit en die analise van mikrobiese gemeenskapstruktuur deur die kwantifisering van spesifieke lipiedbiomerkers, van waarde kan wees as indikatore van grondekologiese stres- of herstelkenmerke omdat dit meer geredelik reageer op klein veranderinge, as fisiese en chemiese eienskappe. Tydens hierdie studie is die verband tussen die fisies en chemiese eienskappe en verskillende biologiese indikatore van grondkwaliteit in die bo-grondlae van sewe steenkoolafvalterreine onder rehabilitasie in Suid-Afrika, asook drie verwysingsterreine, ondersoek. Deur die opname van basiese kwantitatiewe data en die assessering van sekere fisiese, chemiese en biologiese eienskappe van die bo-grondlae, is die relatiewe sukses of vordering van rehabilitasie en die moontlike korrelasie tussen die biologiese indikatore en die vestiging van selfonderhoudende plantbedekking bepaal. 'n Korrelasie is getref tussen die resultate van die fisiese, chemiese en persentasie

plantbedekking-analises en die resultate verkry vir die funksionele en strukturele diversiteit van die mikrobiese gemeenskappe van die onderskeie terreine. Alle resultate is ondersoek met behulp van statistiese en meervoudige-variansie-analises. Die mees prominente fisiese en chemiese parameters wat die biologiese en biochemiese eienskappe van die grond en moontlik die vestiging van selfonderhoudende plantbedekking op hierdie mynafvalterreine beïnvloed, is geïdentifiseer. Resultate van konvensionele mikrobiologiese kwekingstegnieke het geen statisties betekenisvolle ($p>0.05$) verskille tussen die onderskeie steenkoolafvalterreine aangedui nie. Statisties betekenisvolle verskille ($p<0.05$) kon egter waargeneem word tussen die verwysingsterreine. Met die uitsondering van urease en alkaliese fosfatase, het alle ensiematiese aktiwiteite van die rehabilitasie terreine 'n sterk positiewe assosiasie getoon met die organiese koolstof inhoud (%C). Ammoniumkonsentrasie het 'n swak assosiasie met al die toetsensieme getoon, terwyl pH slegs 'n negatiewe assosiasie met suur-fosfatase getoon het. 'n Positiewe assosiasie kon waargeneem word tussen die lewensvatbare mikrobiese biomassa, plantbedekking en die organiese koolstofinhoud, ammonium-, nitraat- en fosforkonsentrasies van die grond. Dit was moontlik om 'n onderskeid te tref tussen die verskillende rehabilitasie en verwysingsterreine op grond van mikrobiese gemeenskapstruktuur soos bepaal deur fosfolipied-vetsuur (PLFA) analises. Daar word van die veronderstelling uitgegaan dat die mikrobiese gemeenskapstruktuur van die Hendrina-terrein nie volhoubaar is wanneer dit volgens 'n r-K gradiënt geklassifiseer word nie en verder, dat die hoë persentasie plantbedekking en hoë vlakke lewensvatbare mikrobiese biomassa 'n kunsmatige weerspieëling is van die huidige bestuurspraktyke wat by hierdie terrein toegepas word. Resultate wat tydens hierdie studie verkry is, dui sterk daarop dat die afwesigheid en/of lae plantbeddekking en geassosieerde laer organiese materiaal in die grond, 'n beduidende negatiewe invloed op die grond-biochemiese eienskappe (ensiematiese aktiwiteit) en grootte van die mikrobiese gemeenskap het. Verder, het die heersende fisies-chemies omgewingsfaktore en bestuurseienskappe 'n beduidende invloed op die plantbedekking en gevolglik op die mikrobiese gemeenskapstruktuur. Die studie dui verder aan dat die mikrobiese ekosisteme van die steenkoolafvalterreine meer stabiel en ekologies selfregulerend kan word, mits effektiewe bestuur toegepas word om te verseker dat die organiese koolstofinhoud van die grond verbeter word. Dit sou 'n verbetering in sirkulering

van nutriënte tot gevolg hê en kan lei tot veranderinge in grondstruktuur en uiteindelik tot verbeterde grondkwaliteit, wat die vestiging van selfonderhoudende plantbedekking sal vergemaklik. Met die resultate verkry tydens die studie, word daar voorgestel dat 'n veelfasige assessering van fisiese en chemiese eienskappe; mikrobiese aktiwiteite deur ensiemanalise; die karakterisering van mikrobiese gemeenskapstruktuur deur die analise van fosfolipied-vetsure; en die meervoudige faktor analise van die data wat verkry is, gebruik kan word as aanvullende assesseringskriteria vir die evaluering van die rigting van rehabilitasie van mynafvalterreine. Strategiese bestuurskriteria word voorgestel gebaseer op die grondkwaliteit/omgewingsvolhoubaarheid-aanwysers om die vestiging van selfonderhoudende plantbedekking te bevorder. Die bydrae van hierdie navorsing tot grondekologie is belangrik met betrekking tot die intensiewe ondersoek en verduideliking van eienskappe en prosesse wat ekologiese rehabilitasie dryf en die kwaliteit van die grondomgewing bepaal. Die multidissiplinêre benadering wat voorgestel word, kan verder bydra tot die suksesvolle rehabilitasie en vestiging van selfonderhoudende plantbedekkings op industriëel versteurde gebiede, asook tot die verbetering van gedegradeerde grondkwaliteit geassosieer met intensiewe en informele landbou. Hierdie benadering kan ook die negatiewe sosiale en omgewingsimpakte wat dikwels met hierdie werksaamhede gepaardgaan, teë werk.

Sleutelterme: *Steenkoolafval; Ensiematiese aktiwiteit; Fosfolipied-vetsure; Grondkwaliteit; Mikrobiese aktiwiteit; Mikrobiese gemeenskapstruktuur; Rehabilitasie.*

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Bmonos	branched monounsaturated fatty acids
Bsat	Base saturation
C	carbon
%C	organic carbon content
$CaCl_2$	calcium chloride
CEC	cation exchange capacity
CFE	chloroform-fumigation extraction
CFI	chloroform-fumigation incubation
CLSU	community level substrate utilisation
CLPP	community level physiological profiles
CO_2	carbon dioxide
CO_3^{2-}	carbonate ion
DGGE	denaturing gradient gel electrophoresis
DGFA	diglyceride fatty acid
DNA	deoxyribonucleic acid
EC	electrical conductivity
EMPR	environmental management progress report
F:B	fungal/bacterial ratio
HCO_3^-	bicarbonate ion
H_2S	hydrogen sulphide
INF	iodonitrotetrazolium chloride formazan
INT	iodonitrotetrazolium chloride
KCl	potassium chloride
Lreq	lime requirement
MBSats	mid-chain branched fatty acids
Monos	monounsaturated fatty acids
N	nitrogen

N_2	molecular nitrogen
NH_3	ammonia
NH_4	ammonium
N_2O	dinitrous oxide
NO_2	nitrite
NO_3	nitrate
Nsats	normal saturated fatty acids
O_2	oxygen
P	phosphorus
PCA	Principal Components Analysis
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
PNG	p-nitrophenyl-β-D-glucosidase
PNP	para-nitrophenol
PO_4^{2-}	phosphate
Polys	polyunsaturated fatty acids
RBS agar	Rose-Bengal Streptomycin agar
RDA	Redundancy Analysis
rRNA	ribosomal ribonucleic acid
S	sulphur
S_2	sulphide
SEA	soil extract agar
SMB	soil microbial biomass
SO_4^{2-}	sulphate
SOM	soil organic matter
TBSats	terminally branched saturated fatty acids
TGGE	temperature gradient gel electrophoresis
TN	total nitrogen
TOC	total organic carbon
Tukey HSD	Tukey Honest Significant Difference
WFPS	water filled pore space

CHAPTER 1

INTRODUCTION

1. THE IMPORTANCE OF MICROORGANISMS IN SOIL ECOSYSTEM PROCESSES

All living organisms depend on three major ecosystems for their survival; that of water, air and soil. Although techniques to assess and ensure the quality of water and air have been in existence for an extended period, there is an ongoing effort to establish an index for soil quality. The fitness of the first two environmental components can be readily assessed because there is no need to attempt an integration of the 'static and functionally dynamic chemical, physical and biological factors defining an ideal state for an infinite number of environmental or management scenarios' as is the case with soil quality assessments (Sojka and Upchurch, 1999). The capacity of soil to function in a manner that upholds vital soil processes depends on the health or quality of that soil. According to Harris and Bezdicek (1994), the terms 'soil quality' and 'soil health' are often used in the same context in literature with scientists generally giving preference to soil quality and producers to soil health. The two terms are also sometimes used without qualification. Use of the term soil health depicts soil as a living, dynamic organism as opposed to soil quality, which rather gives a description of the physical, chemical and biological characteristics (Doran and Safley, 1997). Doran and Safley (1997) defines *soil health* as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health". *Soil quality* is represented by a suite of physical, chemical, and biological properties and the following definition is proposed: "Soil quality is the capacity of soil to function within ecosystem and land-use boundaries, to sustain biological productivity, maintain environmental quality and promote plant, animal and human health" (Doran and Safley, 1997). Similar definitions have been proposed by other

authors in recent literature (Doran and Zeiss et al., 2000; Schoenholtz et al., 2000; Karlen et al., 2003). Evidently, the difference between the definitions for *soil health* and *soil quality* is not a very distinct one and these terms would be used synonymously for the remainder of this work.

In general, the biodiversity of soil exceeds that of aquatic environments or ecosystems aboveground by several orders of magnitude. Soil biota is the 'biological engine of the earth' and microbial groups in particular are of great significance in the maintenance of several fundamental soil processes and ultimately, overall soil quality (Ritz et al., 2003). These include processes of nutrient cycling, maintenance of soil structure, degradation of pollutants and aspects pertaining to human, plant, and animal health (Doran and Zeiss, 2000; Ritz et al., 2003). The major functional processes of ecosystems, namely energy flow and nutrient cycling, interact most strongly in soil. Normally, all energy-consuming processes acquire their energy from photosynthesis. Since there is a lack of photosynthetic organisms in soil, the soil environment does not have the ability to capture solar energy and depends on other sources, such as the energy contained in animal and plant residues and released by means of decomposition (Richards, 1994). Although microorganisms only contribute 0.05% (w/w) of the soil mass (Tate, 2000), they constitute 75-90% of the living fraction of soil (Pankhurst et al., 1997) and microbial activity is fundamental in the processes that make this energy available for recycling in the ecosystem. A number of studies have revealed the crucial roles soil microorganisms play in the biogeochemical cycling of carbon (C), nitrogen (N), phosphorous (P), and sulphur (S) (Bandick and Dick, 1999; Masciandaro and Ceccanti, 1999; Aon and Colaneri, 2001; Marcote et al., 2001). Most nutrients and energy pass through microbial processes first, before being taken up by primary producers, and often this flow of energy and nutrients governs the productivity of the whole ecosystem (Richards, 1994). The relationship between microbial diversity and function in ecosystems is complex and the focal point of much contemporary research. It seems that abundance in diversity *per se* is not automatically the answer to a stable ecosystem because most soil organisms directly mediate more than one function, subsequently leading to potential functional redundancy (Ritz et al., 2003). The concept of redundancy in populations is based on the degree of duplication of function of organisms in

particular ecological processes. It appears that not all processes are unique to particular species; consequently, certain functional aspects in an ecosystem can be maintained even if particular species disappear. Where true functional replication exists, it may be significant in ecosystem maintenance because of increased resilience of a community to species loss and environmental or human perturbations (Hawksworth, 1996). Little redundancy, however, exists in microbially mediated processes, even if there is significant duplication of function in microorganisms. Hence, the loss of functional groups of microorganisms performing essential ecological roles will certainly lead to ecosystem modification or even collapse. The overall species richness of a system will determine the extent to which that system is at risk from the loss of microbial diversity. Disturbances, such as plant, soil and management practices, all have negative impacts on the overall diversity of microbes, for example, the mechanical disruption of soil breaks mycelial stems and reduces the effectiveness of mycorrhizal fungi. Fertilisers, pesticides, and other agrochemicals can significantly alter the diversity and abundance of microbiota (Hawksworth, 1996).

The study of soil organisms is complex for two reasons. The first is the fact that soil organisms, especially microorganisms, are a very diverse group and this makes identification of all individuals an enormous task. Second, there exists an intricate association between many soil organisms and mineral and organic material, so that finding and removing them for further study, can also be very difficult (Ashman and Puri, 2002). It is, nonetheless, essential to pay attention to fields such as soil microbiology and biochemistry in order to predict the ecological consequences of disturbing natural ecosystems and the environmental impacts of utilisation and management practices (Filip, 2002). If changes in microbial community function and structure in an ecosystem are monitored, it can serve as an early warning system of modification to that ecosystem – before damage is reflected in macroorganisms or vegetation. Modifications to an ecosystem may be irreversible or uncontainable by the time it can be observed in the macro-populations, as is currently the practice in most experimental impact assessments. The principal difficulties in biodiversity conservation arise from the lack of knowledge with respect to microorganisms. The fundamental rivets that keep an ecosystem intact are for the most part unknown. Furthermore, the microbial elements of an ecosystem are both

multifaceted and poorly understood. Because of these complex interactions and lack of knowledge, it is questionable whether the rehabilitation of an ecosystem will ensure the preservation of microbial diversity in its original state (Richards, 1994; Hawksworth, 1996).

2. PROBLEM STATEMENT

The South African mining sector provides employment for more than 400 000 people, of which more than 80 000 in the coal mining industry. In 2001 the mining sector accounted for 10% of the countries total gross domestic product (GDP) and 41.5% (R690 billion) of the total market capitalisation of the Johannesburg Securities Exchange. South Africa is the third largest exporter and fourth largest producer of steam coal in the world. In 2001 the coal industries total production amounted to 224 181 171 metric tons and the total sales value to more than R26.5 billion, the third highest of all commodities. Ingwe Mines was responsible for 42 212 941 metric tons of coal sales in 2001, second only to Anglo Operations Ltd (Chamber of Mines of South Africa, 2001).

Even though mining in South Africa contributes to the economy and provides a great deal of employment and training opportunities for local people, the enormous social and environmental impacts caused by mining activities cannot be ignored (Milton, 2001). According to the National State of the Environment Report for South Africa (1999), mining waste constitutes waste rock, tailings (processed material) and polluted process water (DEAT, 1999). Current mining activities generate more than 70 percent of the solid waste produced annually in South Africa (DEAT, 1999). Mine tailings are being processed at a rate of millions of tons per year (Rösner et al., 2001) and discard sites cover large areas of productive land (Van Wyk, 2002). It is thus of great importance to find a sustainable means of mitigating the negative effects associated with mining activities, in the interest of ecosystem health and sustainable land use. Legislation that provides for the restriction of damaging activities to the environment includes the South African Environment Conservation Act (73 of 1989) and the South African Minerals Act (50 of 1991). These laws call for developers to incorporate the cost of ecological rehabilitation into their

operational budgets and require rehabilitation to take place as part of and in conjunction with the mining process. Mine closure and reclamation thus need to be planned from the beginning and executed throughout the mines operation (Hoskin, 2003). According to Hoskin (2003), reclamation is the restoration of land affected by mining to enable, whenever possible, another economic use. In compliance with the mitigation and rehabilitation requirements, many opencast mine rehabilitation projects cover waste rock piles and discard dumps with a layer of topsoil which is excavated from an adjacent borrow pit or stripped from the site before mining (Harris et al., 1989). Agronomic approaches, such as cultivation, fertilisation, reseeding and irrigation have often been adopted for the rehabilitation and revegetation of these sites. This approach has however, failed extensively in the arid areas of southern Africa; primarily due to the lack of the establishment of self-sustainable vegetation cover at these sites, resulting in significant negative environmental consequences (Milton, 2001). The establishment of lasting vegetation cover on mine tailings and discard sites is vital in achieving restoration of these disturbed areas (Carroll et al., 2000). Negative factors that complicate the establishment of vegetation cover include a soil environment typified by poor physical characteristics (Van Wyk, 2002), low levels of plant nutrients and organic matter, pH extremes and the presence of heavy metals (Mining Review Africa, 2003). This is primarily due to the fact that all soil horizons are combined before use as topsoil. In addition, the processing of mine tailings and discard material usually results in an elevated topography which means that these discard sites are particularly exposed to the adverse effects of wind and water erosion (Van Wyk, 2002). These aspects, often accompanied by difficult climatic conditions characteristic to arid and semi-arid areas of southern Africa, deter the establishment of permanent self-sustaining vegetation cover on mine stockpiles and tailings (Milton, 2001; Mining Review Africa, 2003). It is probable that persistent vegetation cover could only be established in conjunction with diverse and self-sustaining biological communities. Clearly, compliance with mitigation and rehabilitation requirements cannot be enforced without a thorough understanding of the ecological principles that ensure ecological stability and subsequent sustainability of ecosystems (Milton, 2001).

Recently, the essence of soil quality in achieving sustainable agronomic, ecological and macro- and microeconomic environments has become apparent, as well as its fundamental role in the establishment of self-sustaining vegetation cover (Masciandaro and Ceccanti, 1999; Marcote et al., 2001). The persistence of vegetation cover on areas under rehabilitative management depends largely on the interaction of revegetated plants with the physical, chemical and biological aspects of the soil profile (Van Wyk, 2002). It is therefore important, when characterising soil quality, to use a selection of all types of soil properties constituting soil quality as a whole. Selected properties should include properties pertaining to chemical, physical and biological aspects of soil and should be those most sensitive to management practices and environmental stress (Hill et al., 2000). Early indicators of changes in soil quality are needed to detect stress and promote long-term sustainability of ecosystems. Chemical and physical parameters change very slowly and therefore many years are required to measure significant changes. On the other hand, soil microbial and biochemical properties are responsive to small changes that occur in the soil, thereby providing immediate and accurate information on the changes in soil quality (Ibekwe et al., 2002).

Soil quality, however, remains difficult to measure because soil and its functions are an ecologically complex phenomenon. It cannot be readily assessed by any single soil parameter, but instead must be evaluated as a function of several independent and/or correlated chemical, physical and biological properties that may exist at different spatial or temporal scales (Doran and Safley, 1997). Industrial and mining companies in South Africa have a social responsibility to ensure that post-land usage capability and subsequently soil quality, should be similar or better than its pre-land use capability as cited in the specific company's environmental management progress report (EMPR). According to Hoskin (2003), the 'objective of mine closure is to leave a mine site in a condition which is safe and stable, and limits further environmental impact so that the mining tenements can be relinquished for alternative land use'. There are certain criteria that have to be adhered to and stipulated as such in the companies Closure Plan. The selection of these criteria must be done in such a manner that a 'balance between costs and benefits of reducing requirements for future care and risk to the environment' is achieved (Robertson and Shaw,

2. Characterisation of the biochemical properties of the topsoil at the various sites by determination of the potential activities of the major enzymes representative of the main steps of soil biogeochemical nutrient cycles, i.e. Carbon (β-glucosidase), Nitrogen (urease), Phosphorous (acid and alkaline phosphatases) and microbial biomass (dehydrogenase activity);
3. Correlation of the physical and chemical characteristics and percentage ground and crown cover of the vegetation growing at the various sites, with the functional and structural diversity of the microbial communities;
4. Multivariate statistical analysis of the influence of the dominant soil physical and chemical characteristics on the microbial community function and structure;
5. Identification of the predominant physical and chemical parameters that influence the biological and biochemical properties of the soil and subsequent self-sustainability of vegetation cover on these mine-tailing sites; and
6. Recommendation of strategic management criteria for the manipulation of operational criteria based on the soil quality/environmental sustainability indices, to facilitate the establishment of self-sustainable vegetation covers at the coal discard sites.

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CHAPTER 2

LITERATURE REVIEW

1. THE SOIL ECOSYSTEM

All ecosystems exist as mosaics of interrelated and mutually dependent properties and processes. Beedlow et al. (1988) described an ecosystem as a self-ordering biotic-abiotic system that has developed a homeostatic state over time. The abiotic portion of soil consists of minerals, organic matter, soil water and soil atmosphere. The inorganic material is in the form of a mineral fraction that is described by the different sized particles, their chemical composition and cation exchange capacity (CEC) (Richards, 1994). The term 'soil organic matter' (SOM) includes, in its widest sense, all the living and dead organisms contained in soil and is the result of decomposition and incorporation of plant and animal residues into the soil. Carbon is the main constituent of SOM and the concentration of soil carbon is often used as a measure of SOM content (Ashman and Puri, 2002). The greater part of the organic matter in soil is adsorbed onto clay surfaces (the clay-organic complex); the rest is plant material yet to be decomposed (Richards, 1994). Table 1 shows some typical soil carbon and nitrogen concentrations.

Table 1. Broad soil carbon and nitrogen ratings, expressed as percentage values (Ashman and Puri, 2002).

Rating	Carbon (%)	Nitrogen (%)
Very high	>20	>1.0
High	10-20	0.5-1.0
Medium	4-10	0.2-0.5
Low	2-4	0.1-0.2
Very low	<2	<0.1

Soil pores are occupied by either water or air. Water is held within the structural components of the soil by forces that lower the water potential; the water potential is also lowered by solutes. The greater the pore volume of the soil, the more water can be held – both in spaces between macro- and micro pores and adhered to soil particles. A variety of solutes, such as nutrient ions, contained in soil water is required by microorganisms and plants to successfully execute a range of metabolic functions (Rowell, 1994; Madigan et al., 1997). The manner in which soil particles are aggregated, i.e. their structure, greatly influences the processes that occur in the soil environment. Soil structure is determined by the nature of soil aggregates, which are random combinations of soil organic and mineral components assembled into micro- (<50 μm mean diameter) and macroaggregates (>50 μm mean diameter particles). These aggregates are a product of the interactions of the soil microbial community, soil parent material, aboveground vegetation and ecosystem history. The metabolism of soil microorganisms can alter soil structure; for example, the production of polysaccharides enhances soil structure by linking more soil particles into macroaggregates. Furthermore, soil microbial metabolism is greatly influenced by the association between the soil particles to which the microorganisms are attached and the larger soil aggregates (Tate, 2000). This can be attributed partly to the fact that the degree of soil aggregate formation controls soil properties such as water infiltration and availability, oxygen tension and nutrient movement. Generally, well-structured soils are better aerated and microbial and root respiration can occur more freely (Rowell, 1994; Tate, 2000).

The biotic portion of the soil ecosystem is composed of living biomass that includes plant roots, fauna and microorganisms (Pankhurst, 1997). Table 2 gives a representation of the composition of the functional groups of soil fauna and microorganisms found in a typical fertile soil. The average percentage dry weight of the living biomass is also indicated.

Table 2. Composition of a typical fertile soil in terms of its biota and functional groups of microorganisms. Numbers are percentage dry weight (Adapted from Pankhurst, 1997).

	Biomass	**Trophic/functional groups**
Roots	5-15%	
Fauna	5-10%	Protozoa, nematodes, earthworms, microarthropods
Microorganisms	75-90%	Decomposers, N_2-fixing microorganisms, denitrifiers, mycorrhizae, algae

The primary source of energy and carbon for microorganisms enters soil as plant biomass and root exudates, making the rhizosphere the site of maximum biological activity (Tate, 2000). Exudation is not metabolically mediated and exudates are compounds of low molecular weight (e.g. monosaccharides and amino acids) that leak from all cells into the soil either directly or via intracellular spaces (Richards, 1994). The composition of root exudates selectively stimulates microbial populations and is a primary parameter in selecting for individual species active in the rhizosphere community (Tate, 2000).

2. SOIL MICROBIOTA

The smallest group of soil organisms (<200 μm) is termed the 'microbiota' and includes viruses, bacteria, fungi, protozoa and algae (Atlas and Bartha, 1998; Ashman and Puri, 2002). Table 3 shows representative numbers of individuals per gram of soil for five major groups of the microbiota.

Table 3. Approximate numbers of organisms (per gram) commonly found in the microbiota (Ashman and Puri, 2002).

Organism	**Estimated no./g**
Bacteria (not including Actinomycetes)	$3 \times 10^6 - 5 \times 10^8$
Actinomycetes	$1 \times 10^6 - 2 \times 10^7$
Fungi	$5 \times 10^3 - 9 \times 10^5$
Algae	$1 \times 10^3 - 5 \times 10^5$
Protozoa	$1 \times 10^3 - 5 \times 10^5$

Bacteria and fungi are the two major groups of microorganisms involved in soil metabolism. Both groups make use of the hydrolysis of complex compounds by exoenzymes to degrade insoluble substrates. Fungi however, are better equipped for the breakdown of cellulose because of their intrinsic mechanism for penetrating plant tissues. The combination of mechanical pressure from the hyphae and the action of exoenzymes, makes breakdown of their substrate much more effective than when using exoenzyme action alone (as in the case of bacteria). It is only in anaerobic environments that cellulolytic bacteria dominate cellulolytic fungi (Richards, 1994).

2.1. Bacteria

Numerous genera of bacteria occur in very high individual numbers in soil, especially in the rhizosphere, and they are able to perform an array of functions in the soil environment. Soil bacteria tend to be attached to soil particles - an association that can be related to the buffering capacity of clay minerals and the higher concentrations of nutrients found on clay surfaces (Atlas, 1997; Ashman and Puri, 2002). This association between soil bacteria and soil particles is probably the major limiting factor in extracting them from the soil environment without compromising their morphology and metabolism (Ashman and Puri, 2002). Soil aggregates may contain a number of microhabitats and therefore several types of microorganisms. Soil bacteria exhibit a number of metabolic strategies, including aerobic, facultative anaerobic, microaerophilic and obligate anaerobic metabolism. Bacterial genera that make up a large proportion of the microbial community in soil include the Gram-positive rods *Arthrobacter* and *Corynebacterium*, and the aerobic sporeformer *Bacillus*. The Gram-negative rods *Pseudomonas* are also active in organic matter decomposition and have great biochemical versatility, being able to use a far wider range of organic compounds as carbon and energy sources than any other group of microorganisms. The cyanobacteria are another group of bacteria found in soil and aquatic and terrestrial forms are included in this group. Some species, such as *Azotobacter*, can convert atmospheric nitrogen to fixed forms of nitrogen (Richards, 1994; Atlas and Bartha, 1998).

2.2. Fungi

Fungi are essential to soil quality for a number of reasons: they represent large nutrient pools and an important food source for other soil organisms; they contribute to soil aggregate stability; and play a significant role in the decomposition and mineralisation of organic residues (Stahl et al., 1999). They are chemotrophic microorganisms that depend on chemical sources of energy for their life processes and absorb their nutrients from solution (osmotrophs). Fungi are especially prominent in forest ecosystems where low soil pH often restricts the activities of bacteria (Richards, 1994). In terms of biomass, fungi are the dominant microbial group in most soils. Adverse environmental conditions can be overcome by forming spores or by producing mycelial cords to assist in the spread of nutrients and water. Fungi flourish under somewhat lower pH conditions, particularly where plant residues contain high concentrations of lignin (Ashman and Puri, 2002). Ascomycetes and imperfect fungi, notably *Penicillium, Fusarium, Aspergillus* and *Trichoderma,* and zygomycetes such as *Mucor* and *Rhizopus,* are among the fungi most frequently isolated on soil dilution plates. Some fungi are associated with plant roots (mycorrhizae) and are very difficult to isolate and identify (Atlas and Bartha, 1998). Mycorrhizal fungi are incapable of decomposing organic matter, yet they play a significant role in the energy cycles of an ecosystem. Their importance in nutrient cycling and decomposition makes them irreplaceable in the maintenance of soil fertility (Alef and Nannipieri, 1995; Hawksworth, 1996). These fungi form ectomycorrhizae – a symbiotic relationship between mycorrhizal fungi and plant roots. The plants supply the fungi with photosynthate and the fungi provide mineral nutrients to the plants. The result is enhanced plant growth, more deposition of leaf litter on soil and greater amounts of root detritus; all of which result in a greater amount of energy being returned to the system despite the fact that the fungi are not able to directly release energy from decomposition (Richards, 1994).

3. THE ROLE OF MICROORGANISMS IN ECOSYSTEM PROCESSES

Microorganisms are interlaced into all the systems that support life on earth but most terrestrial microbes are found in soil (Hawksworth, 1996). Terrestrial environments mostly

pertain to soil and plants (Madigan et al., 1997) and the diversity in habitats that microbes occupy is the consequence of variations in soil properties such as moisture, aeration, temperature, pH, and nutrient supply (Rowell, 1994). Even though soil microorganisms constitute less that 0.5% (w/w) of the soil mass (Tate, 2000), they represent much larger numbers and biomass than microorganisms occurring elsewhere, such as on the surfaces of plants. Together with exocellular enzymes and soil macro- and mesobiota, they conduct all known metabolic reactions in the soil they inhabit. They produce trace gasses, such as methane; help regulate populations when used as biocontrol tools; play unique roles in the circulation of matter, such as nitrogen fixation; and are part of the food chains and food webs on which all macro-organisms depend. Other ecosystem processes in which different groups of microorganisms are involved include soil stability and structure; decomposition of plant and animal remains and products; rock weathering (Richards, 1994; Hawksworth, 1996; Ashman and Puri, 2002); suppression of pathogenic microorganisms; and detoxification of pollutants (Snakin et al., 1996). Physical and chemical soil properties, such as pH, cation exchange capacity, salinity, solubility of soil mineral components and aggregate structure are constantly being altered by the activities of soil microorganisms (Tate, 2000).

3.1. Energy flow and organic matter decomposition

Microbial communities require energy and nutrients in order to maintain their structure and function. Nutrients are not distributed equally throughout the soil environment; there is a decreased concentration in elements such as nitrogen, phosphorus, calcium and sulphur from the surface to the deeper soil layers. Variation also exists in horizontal patterns, mainly due to vegetation and the consequent differences in passage of nutrients to soil. Nutrients needed in addition to energy and carbon sources, are referred to as growth factors. Those microorganisms that do not require growth factors must have all the enzymatic systems needed to be able to synthesize the organic compounds they require during metabolism. The limits between which microbial species are able to grow optimally, define their ecological tolerance for a specific environmental factor, and any factor that tends to slow down the growth of the organism is referred to as a limiting factor. There is a

minimum level below which the organism will not grow at all, an optimum level at which growth is best and a maximum level above which again no growth occurs (Richards, 1994; Atlas and Bartha, 1998). These abiotic limitations to microbial growth regulate or exclude the existence of microorganisms in various environments and are described by Liebig's *law of the minimum*. According to this law the 'total yield or biomass of any organism will be determined by the nutrient present in the lowest (minimum) concentration in relation to the requirements of that organism' (Atlas and Bartha, 1998). In other words, the affected population would grow or reproduce if there is an increase in the concentration of a particular limiting nutrient, until another factor becomes limiting. Furthermore, populations in the same ecosystem may be limited by different limiting factors. In this case, the addition of nitrogen, for example, would allow the growth of one population of microorganisms (nitrogen is the limiting factor), while another population would not grow (nitrogen is not the limiting factor) (Atlas and Bartha, 1998).

Shelford's *law of tolerance* pertains to abiotic limitations other than nutrients. It states that environmental conditions exist above or below which microorganisms cannot survive. Therefore, conditions must remain within a tolerance range in order for a given organism to be successful in its environment. Factors that can be limiting in this context include physical and chemical determinants such as temperature, redox potential, pH, hydrostatic pressure and salinity (Atlas and Bartha, 1998). Table 4 gives some extreme physiological tolerance limits for microbial activity.

Table 4. Some extreme physiological tolerance limits for microbial activity (Atlas and Bartha, 1998).

Factor	Lower tolerance limit	Upper tolerance limit
Temperature	-12°C	>110°C
	(psychrophilic bacteria)	(sulphur-reducing bacteria at 1000atm; sulphur oxidisers in deep-sea thermal vent regions)
E_h (redox potential)	-450mV	+850mV
	(methanogenic bacteria)	(iron bacteria)
pH	0	13
	(*Thiobacillus thiooxidans*)	
Hydrostatic pressure	0	1400 atm
	(various microorganisms)	(barophilic bacteria)
Salinity	0	Saturated brines
	(*Hyphomicrobium*)	(*Dunaliella*, obligate halophilic bacteria)

The occurrence of any group of microorganisms in a given environment is thus dependent on the nutritional requirements (Liebig's *law of the minimum*) and environmental tolerance (Shelford's *law of tolerance*) of those organisms. It is also important to realise that environmental tolerance is influenced by the interactive nature of different parameters. For example, a microorganism might not be able to survive at a particular hydrogen ion concentration and particular temperature in a specific ecosystem, but would proliferate at the same hydrogen ion concentration and a different temperature in another ecosystem (Atlas and Bartha, 1998).

The productivity of an ecosystem is measured by the amount of organic matter fixed per unit of time (Richards, 1994) and is frequently the limiting factor for growth of heteroptrophic microorganisms (Atlas and Bartha, 1998). The processes of accumulation and decomposition of organic matter is strongly comparable with energy flow through the system (Richards, 1994). Furthermore, SOM is central to the maintenance of soil fertility because it affects structural stability, water-holding capacity and mineralisation of important elements such as nitrogen and phosphorus (Rowell, 1994). Soil organic matter can only begin to accumulate in the soil once certain bacteria, fungi and plant species, known as primary colonisers have colonised mineral particles. These organisms (such as

nitrogen fixers) have the capacity to acquire nutrition from sources other than the soil and can therefore live in otherwise hostile environments. Primary colonisers make it possible for other organisms to grow because they increase the amount of SOM present in the soil (Ashman and Puri, 2002). Only a fraction of the total SOM participates in the mineralisation-immobilisation cycle at one time. The greater part of organic matter that enters the soil is more or less stable against microbial attack once it is decomposed. Environmental factors, such as temperature and alternate cycles of wetting and drying, greatly influence organic matter decomposition (Richards, 1994).

3.2. The biogeochemical cycling of elements

Atlas (1997) defined biogeochemical cycling as "the movement of materials via biochemical reactions through the global biosphere". The chemical transformation of elements results in the physical translocations of materials, in other words the exchange of elements between the atmosphere, hydrosphere and lithosphere and is essential for all forms of life on earth. Microorganisms play a vital role in these cycles because they are capable of decomposing every naturally occurring organic material known to exist (Atlas, 1997) and without this continuous recycling of nutrients, soil would become barren (Ashman and Puri, 2002). Some microorganisms are very specific as to the compounds they decompose; others decompose a wider range. The decomposition of organic wastes also depends on the activities of microbes and when microbial decomposition is ineffective, organic compounds accumulate, such as in peat lands. The main biogeochemical cycles include the carbon, nitrogen, phosphorus and sulphur cycles (Atlas, 1997). Although these cycles are discussed separately, it is important to bear in mind that they are interlinked and dependent on each other, with many reactions occurring simultaneously.

3.2.1. Carbon cycling and the role of active and inactive microorganisms

Carbon (C) is cycled through ecosystems by a combination of carbon fixation by autotrophs and decomposition by heterotrophs. It cycles in the form of inorganic carbon dioxide (CO_2) and various organic compounds. Of all the elements required by

microorganisms, carbon is required in the greatest amounts. Carbon compounds can be found in every living organism and the cycling of carbon is crucial to the functional processes of all these organisms. Inorganic carbon in soil is found mainly as carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-). Chemical erosion of rocks, biogenic deposits (such as coal and petroleum), and humus are all mechanisms in which carbon is supplemented into the soil environment (Richards, 1994; Atlas, 1997). Microorganisms can metabolise both organic and inorganic carbon and the autotrophic metabolism of photosynthetic and chemolitotrophic microbes is responsible for the conversion of inorganic CO_2 to organic carbon. Carbon can be transferred from one population to the next once it is reduced to organic compounds. This supports the growth of many heterotrophic organisms. The oxidation of organic compounds is the means by which most chemotrophic bacteria obtain energy and carbon. Nitrifying bacteria only use inorganic substances for their chemoautotrophic metabolism, and are thus dependent on atmospheric CO_2 as their sole carbon source. Inorganic CO_2 is returned to the atmosphere by the respiration and fermentation reactions of heterotrophic organisms (Richards, 1994; Atlas, 1997). Chemoheterotrophic microorganisms that decompose organic matter and mineralise carbon are not distributed evenly through soil. Their activities are influenced by the supply of substrates, which is the major limiting factor. The more carbonaceous materials present in the soil, the greater the number and activity of chemoheterotrophs will be. The upper part of the soil profile is usually the habitat of these microorganisms. This distribution of microbes is not only limited by the availability of organic matter (Ashman and Puri, 2002), but also by other factors. The most apparent is the depletion of oxygen (O_2) and higher concentration of CO_2 deeper into the soil layers (Richards, 1994).

The lack of sufficient carbon inputs in most soils has led to the assumption that microorganisms exist at different levels of activity, with only a fraction of the microbial biomass being active (Ashman and Puri, 2002). This occurrence provides microorganisms with a strategy (r-K strategy) to ensure their continued existence, based on presumed differences in their ability to exploit resources and survive in different environments. Two groups are distinguished: r strategists prevail in unstable environments, while K strategists generally favour stable environments (Sarathchandra et al., 2001). The first group, also

referred to as the zymogenous population, are opportunistic microorganisms with high rates of reproduction in response to substrate inputs. Heterotrophs, such as *Penicillium, Pseudomonas,* and *Bacillus* are typical r strategists. The K strategists are autochtonous, exhibiting greater population stability because they are characterised by slow growth and death rates and can grow successfully under conditions of low substrate availability. Included in this group are the soil streptomycetes, *Agrobacterium, Corynebacterium* and similar humus-degrading soil bacteria. Typically, r strategists compete better at low population densities because they have few competitive adaptations besides a rapid growth rate and could therefore be characteristic of populations initially colonising a habitat. On the other hand, K strategists reproduce slowly and depend on physiological adaptations and the carrying capacity of the environment to survive. It is presumed that generally the soil microbial biomass consists of a small active population and a larger, inactive population (Atlas and Bartha, 1998; Ashman and Puri, 2002).

3.2.2. The role of microorganisms in the nitrogen cycle

Nitrogen (N) is required in large amounts by organisms to provide for the synthesis of amino acids, proteins, nucleotides and vitamins. It occurs in various oxidation states in nature, specifically ammonium (NH_4), nitrate (NO_3), nitrite (NO_2), and molecular or atmospheric nitrogen (N_2), which is the most abundant form of nitrogen in the atmosphere. Most organisms, however, cannot utilise N_2 – only a few microorganisms, known as nitrogen fixers, have this capability. Nitrogen fixers incorporate N_2 into the various soil nitrogen pools where it is made available in other forms of nitrogen to a variety of microorganisms (Rowell, 1994; Atlas, 1997). The activities of microorganisms and plants result in the continuous movement of soil nitrogen from one form to another. Microorganisms are responsible for processes of mineralisation, immobilisation, nitrogen fixation, nitrification and denitrification (Rowell, 1994).

Accumulated ammonium in soil represents the quantity of substrate nitrogen in excess of microbial requirement, because ammonia (NH_3) is a byproduct of microbial metabolism (Richards, 1994). Nitrogen in the form of ammonium is used by photoautotrophs,

chemoheterotrophs and a few bacterial species of chemoautotrophs that carry out nitrification. During this two-stage process, NH_4^+ is first oxidized to NO_2^- and then to NO_3^- (Atlas, 1997; Atlas and Bartha, 1998). Table 5 shows the genera of nitrifying bacteria that carry out these two reactions and the environments in which they occur.

Table 5. Genera of nitrifying bacteria and the environments in which they occur (dominant genera are indicated in bold print) (Adapted from Atlas, 1997).

Conversion	Microorganism	Environment
NH_4^+ to NO_2^-	***Nitrosomonas***	Soil, freshwater, marine
	Nitrosospira	Soil
	Nitrosococcus	Soil, freshwater, marine
	Nitrosolobus	Soil
NO_2^- to NO_3^-	***Nitrobacter***	Soil, freshwater, marine
	Nitrospira	Marine
	Nitrospina	Marine
	Nitrococcus	Marine

Plants and microbes use nitrate as a source of nitrogen in a similar way to ammonium and it undergoes a series of microbially mediated processes until it is returned to the atmosphere in the form of N_2 by denitrification (Ashman and Puri, 2002). The accumulation of ammonium depends on the ratio in which carbon and nitrogen is supplied to the soil. The influence of the C/N ratio on mineralisation and immobilisation can be modified by environmental factors. Decomposition is slower under low temperatures and anaerobic conditions, as opposed to higher temperatures and aerobic conditions. Less nitrogen is needed when the rate of decomposition is lower (Richards, 1994; Ashman and Puri, 2002). Nitrogen is also returned to ecosystem in the form of urea, which is a natural product of animal excretion and is constantly added to the environment. The hydrolysis of urea by heterotrophic soil bacteria liberates mineral nitrogen that is taken up by plants and microorganisms and converted into organic nitrogen (Richards, 1994; Rowell, 1994).

3.2.3. Phosphorus cycling in soil

Phosphorus (P) occurs in soils in various fractions, including phosphate minerals, attached to particle surfaces and in soil solution, plant phosphorus, and organic matter. Compared to nitrate, concentrations of phosphate (PO_4^{2-}) in soil are much lower – the result of the low solubility of phosphate minerals and strong adsorption of phosphate onto particle surfaces (Rowell, 1994).

Organisms require phosphorus (usually in the form of PO_4^{2-}) in considerable amounts as a component of nucleotides and nucleic acids, as well as phytates and phospholipids. Most of this phosphate is of mineral origin (inorganic phosphate) but some is derived from the enzymatic breakdown of inositol hexaphosphates in SOM by phytase-producing microbes (Richards, 1994). Phosphatase enzymes catalyse the release of inorganic phosphorus from organic-bound phosphorus returned to soil as plant residues and other organic debris. This activity plays an important role in the phosphorus cycle (Garcia et al., 2002). Another important phenomenon in making phosphorus available to the ecosystem involves mycorrhizae. Phosphate uptake by roots is small and diffusion rates slow due to adsorption and low solution concentrations, hence an extensive root system is essential for sufficient nutrient uptake. A symbiosis with mycorrhizae would therefore be of great advantage to plants as the mycorrhizae grow out from the roots into the soil, take up phosphorus and other nutrients and transport it to the root in return for a supply of carbohydrate (Rowell, 1994; Entry et al., 2002). Mycorrhizae will not, however, improve phosphorus uptake in soils with a large concentration of extractable phosphorus because the root demand is easily satisfied by diffusion from the soil (Rowell, 1994).

3.2.4. The sulphur cycle

Sulphur (S) is a mineral nutrient essential to microbial metabolism and is, similar to nitrogen and phosphorus, cycled in a complex oxidoreductive manner. Sulphur is assimilated in the form of sulphate (SO_4^{2-}) by plants, algae and many heteroptrophic microorganisms (Atlas and Bartha, 1998). In the absence of light, *Thiobacillus* and other

litoautotrophs use sulphide (S_2) as an electron source during oxidative sulphur transformations (Nicklin, et al., 1999). Microaerophilic bacteria, including *Beggiatoa, Thioploca, Thiothrix,* and *Thermothrix,* also oxidise hydrogen sulphide (H_2S) (Atlas and Bartha, 1998). Another manner in which microorganisms can carry out sulphate transformations, is sulphate reduction. Two types of reduction can be distinguished. The use of sulphate as an external electron acceptor to form sulphide is described as dissimilatory reduction. Assimilatory reduction, on the other hand, involves the reduction of sulphate for amino acid and protein biosynthesis (Nicklin, et al., 1999).

4. THE IMPORTANCE OF SOIL QUALITY IN THE MAINTENANCE OF ECOSYSTEM HEALTH

Soil is a crucial component of the biosphere and soil quality is intrinsically linked to overall environmental quality and ultimately, sustainability (Marcote et al., 2001). In recent years this realisation and the growing awareness of perturbations in the soil environment has resulted in research focussed on the sustainability of soil health (Dick et al., 1996). Soil quality is determined by a number of physical, chemical and biological factors that influence each other and the overall state of quality in the soil ecosystem (Karlen et al., 2003). Although it is difficult to define soil quality, there seems to be, for the most part, agreement between authors in the literature that 'soil quality' includes a measure of a soil's capacity to function within an ecosystem and sustain equilibrium through certain processes. Soil functions to produce plant biomass, maintain animal and human health, recycle nutrients, store carbon, partition rainfall, buffer anthropogenic acidity, remediate added animal and human wastes, and regulate energy transformations (Doran and Safley, 1997; Pascual et al., 2000; Schoenholtz et al., 2000; Ruf et al., 2003).

Soil, water and atmospheric quality have remarkable influences on each other. However, this interrelationship is often overlooked. Intensive industrial and agricultural practices, such as tillage, cropping patterns and the application of pesticides and fertilisers are responsible for adverse effects on the quality of surface and sub-surface water worldwide. Consequently, there is an imbalance of carbon, nitrogen and water cycling in soil (Doran,

2002; Karlen et al., 2003). Additionally, air quality can be compromised due to changes in the capacity of the soil to process important atmospheric gasses (carbon dioxide, nitrous oxide, methane), contributing to the problem of global climate change and ozone depletion (Bengtsson, 1998).

One of three different temporal trends can be observed in soil quality assessments (Figure 1).

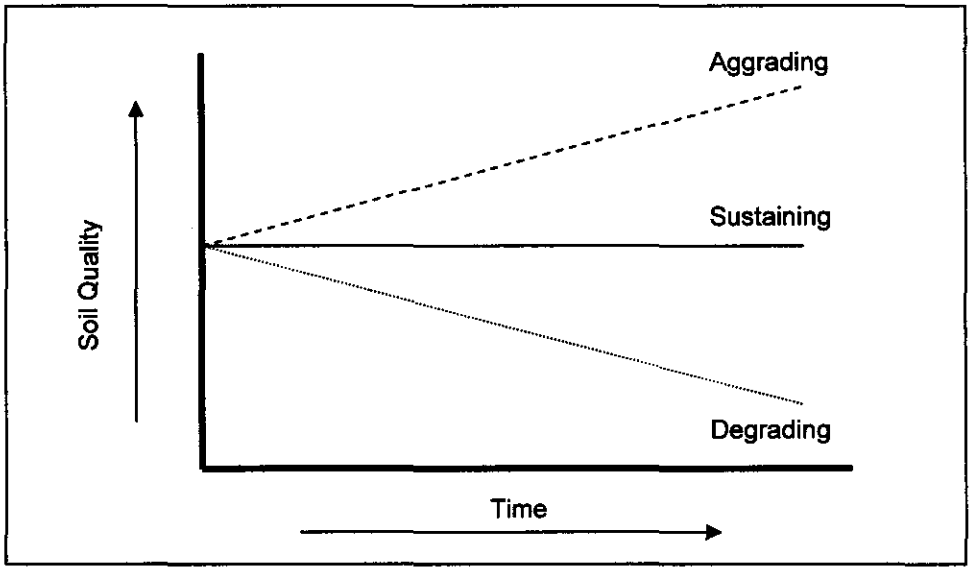

Figure 1. Possible temporal trends in dynamic soil quality assessments (Karlen et al., 2003).

Soil can either be in a degrading, sustaining or aggrading state (Karlen et al., 2003). In a degrading state, the soil function would be impaired, while in an aggrading state a trend of enhanced function would be evident. Beedlow et al. (1988) described an ecosystem capable of sustaining its homeostatic state (within bounds), as stable and furthermore, that this stability could be attributed to the system being either resilient or resistant or a combination of both. Resilience refers to the ability of the system to return to a state of equilibrium after it has been disturbed, in other words, to regain functional and structural characteristics that may have been subjected to stress or disturbance. On the other hand, a resistant system maintains functional and structural equilibrium under conditions of stress or disturbance (Figure 2) (SER, 2002; Ritz et al., 2003).

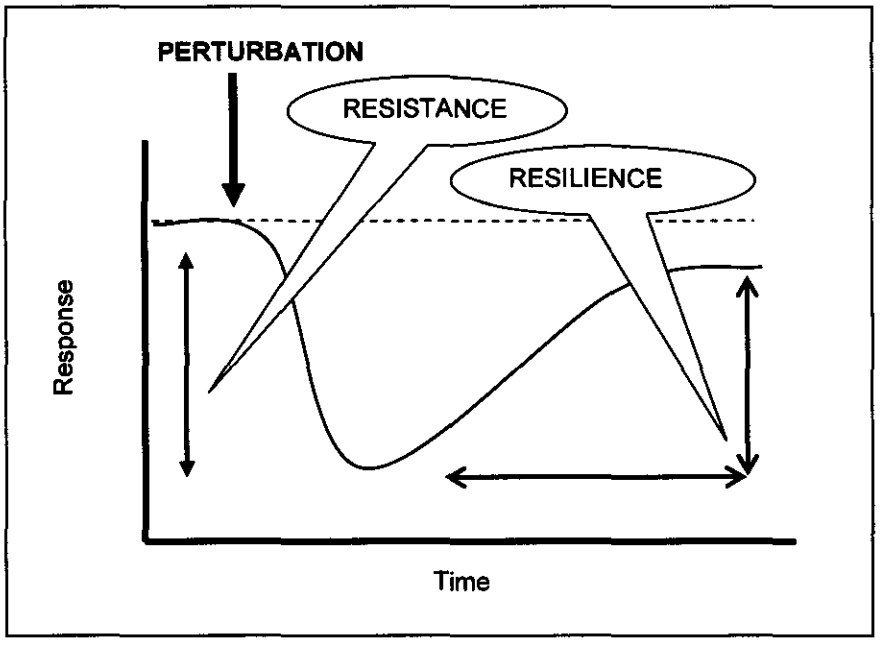

Figure 2. Graphical representaion of trajectory of resistance and resilience in perturbed systems. Broken line shows timecourse of response variable in unperturbed (control) systems, solid line shows response following perturbation. Resistance is measured as the degree of impairment of response relative to control; resilience as the rate and extent of recovery. Recovery may be incomplete within the measured timescale (Ritz et al., 2003).

Another important function of resilience is its role in the recovery process of an ecosystem. This is the process by which a system returns to its original or to a new state of equilibrium following disturbance (Beedlow et al., 1988). Since disruptions to the soil environment is such a regular occurrence, it is obvious that resilience and/or resistance in soil ecosystems are crucial in order for these systems to maintain a certain degree of normal functionality.

According to Karlen, et al. (2003) the focus of soil quality and its assessment includes two important principles: 'These are that (1) soil quality is determined by both inherent and dynamic properties and processes interacting within a living, dynamic medium, and (2) that it is holistic, reflecting biological, chemical and physical properties, processes, and interactions within soils'. Despite many studies on the subject of soil quality, a distinct definition and comprehensive set of indicators are still lacking (Bouma, 2002). What is certain is that the quality of a soil is mostly determined by natural soil composition and

anthropogenic activities. Worldwide, processes of erosion, anaerobiosis, salinisation, compaction, organic matter depletion and nutrient imbalance are responsible for the lowering of soil quality and health (Schoenholtz et al., 2000). Poor management practices and other negative impacts result in the loss of functional groups and subsequent ecosystem modification or even collapse (Hawksworth, 1996).

5. ECOSYSTEMS AT RISK FROM MICROBIAL DIVERSITY LOSS

The past half a century has seen considerable losses in terms of soil quality worldwide. Of the 8.7 billion hectares of agricultural land, permanent pastures and forests and woodlands, around 2 billion hectares have been degraded (Arshad and Martin, 2002). Soil is a vital natural resource and is non-renewable on a human time-scale (Doran and Safley, 1997; Tate, 2000). In view of the fact that the importance of soil as a natural resource has been recognised, serious concerns exist pertaining to intensive land-use that causes environmental degradation (DEAT, 1999). The depletion of natural resources that result in the transformation and fragmentation of natural habitats leads to changes in the number and type of species that occur there and inevitably, to impaired ecosystem functioning (Hawksworth, 1996; DEAT, 1999). Soil microbial communities are critical to the ecological functioning of terrestrial ecosystems (Bandick and Dick, 1999; Aon and Colaneri, 2001) and because they are so sensitive to changes in their chemical and physical environment (Ibekwe et al., 2002), disturbance of the soil ecosystem is potentially detrimental to short- and long-term stability (Mummey et al., 2002a,b).

5.1. The State of Terrestrial Ecosystems in South Africa

According to the National State of the Environment Report for South Africa (1999), 25% of South Africa's land area has been transformed, of which 13% is for cultivation of crops, 1.5% for forestry and 2.5% to settlements (DEAT, 1999). Five percent has been degraded, and only 6% is under formal protection. Over 8% of South Africa has been invaded by alien vegetation, and millions of hectares are affected by bush encroachment. Five per cent of soils are affected by water erosion, and the average soil loss is 2.5 tonnes per hectare per

year. Soil formation is estimated at 0.31 tonnes per hectare per year, which would mean that the current rate of soil loss in South Africa is more than eight times the rate of soil formation – a situation that is obviously unsustainable (DEAT, 1999).

5.2. The Impact of Environmental Disturbance on Soil Quality

Ecosystems are not only disturbed by anthropogenic influences, sometimes natural disturbances have similar effects to human-induced impacts. These include fires caused by lightning; intensive grazing by wild animals; and landslides, that is comparable to construction activities (Beedlow et al., 1988). Past management practices of agriculture and other ecosystems to meet the needs of escalating populations has put enormous strain on the resilience of soil and natural processes to maintain global balances of energy and matter (Doran and Safley, 1997). Population growth, industrialisation, urbanisation and intensive agricultural and forestry production are continuously responsible for large amounts of waste and imminent pressure on terrestrial ecosystems (DEAT, 1999). Soil degradation includes physical degradation (compaction, crusting, structural deterioration, erosion, desertification); chemical degradation (acidification, salinisation, sodicity and alkalination, nutrient depletion, pollution, toxicity); and biological degradation (decline in SOM, loss of biodiversity, and soil sterility) (Snakin et al., 1996; DEAT, 1999). As degradation occurs, some soil properties change, particularly soil microbial activity, which is fundamental in the maintenance of soil quality (Garcia et al., 2002). Disturbance can be characterised in terms of intensity, scale, duration, seasonality and type. It is these characteristics that will determine which of the various soil ecosystem processes will be impacted (Beedlow et al., 1988). Some important negative impacts on soil quality are discussed.

5.2.1. The implications of soil compaction on the soil environment

Industrial and agricultural activities and other compactive processes cause disruption of the soil structure, which results in an increase in the soil density. Water movement through the soil is subsequently hindered, causing saturation and waterlogging in or above the compacted layer (Snakin et al., 1996), as is the movement of water aboveground, with

increases in runoff (Soane and Van Ouwerkerk, 1995). Soil saturation alters the hydrological, physical and chemical soil parameters. This has drastic effects on microbial function because of resultant anaerobic conditions (Snakin et al., 1996) and changes in the demand and supply of plant nutrients. Anaerobic soil conditions favour nitrification, denitrification and an increased release of nitrates from soil. Compaction results in changes in soil permeability and aeration and may change fluxes of greenhouse gases from the soil to the atmosphere. The ability of soil to filter out various toxic substances, such as those arising from the breakdown of pesticides, is another function that may suffer due to compaction and will compromise the quality of surrounding water supplies (Soane and Van Ouwerkerk, 1995).

5.2.2. Fire as a negative impact

Although many studies have emphasised the importance and benefits of regulated forest and veldt fires, it is important to realise that the ecosystem as a whole may not always benefit from fire. Fires can be extremely destructive to vegetation cover and subsequently interfere with normal functions of the ecosystem, e.g. the maintenance of water supplies, protection against erosion and the accumulation of nutrients (Saá et al., 1996). When soil is altered in this way, it decreases the amount of organic matter and mineral nutrients in the soil (Hungerford et al., 1991). A study by Saá et al. (1996) demonstrated that when the top 0-5 cm layer of soil is exposed to the heat that accompanies a forest fire, it causes the oxidation of organic phosphorus and a marked decrease in the content of residual phosphorus. This results in a loss to the ecosystem of a reservoir of phosphorus for plant nutrition. Other negative effects of fire include the following: denaturing or inactivation of enzymes crucial to ecosystem processes (Saá et al., 1996); loss of stored water and amino acids; volatilisation of nitrogen and sulphur; reduced soil porosity, water movement and water holding capacity; and death of plant tissue and seeds. Microorganisms are affected directly by heat and indirectly by physical and chemical changes in soil (Hungerford et al., 1991).

5.2.3. The effect of plant cover decline on soil parameters

The decline in plant cover in general, often because of fires, overgrazing and the extensive removal of firewood from many forests, have led to the overexploitation of such forests and as a result, the duration of fallow periods has been significantly reduced. Reduced fallow periods are also the result of intensive agricultural practices. Fallow periods are essential for the regeneration of vegetation, leading to the restoration of soil properties (Badiane et al., 2001; Garcia et al., 2002). A degraded plant cover means a lower SOM content, which leaves the microorganisms less organic matter to decompose. Plant cover decline is also linked with changes in the nitrogen cycle, lower urease and protease activity (Garcia et al., 2002) and considerable soil losses by wind and water erosion (Castillo and Joergensen, 2001).

Virtually 91% of South Africa is situated within the United Nations definition of 'affected drylands' (UNCCD, 1994). These terrestrial ecosystems are extremely dry areas where rainfall is low and potential evaporation is high. Dryland systems are fragile and need to be managed carefully. The loss of vegetation ground cover from such areas poses an increased risk of erosion, the outcome being less fertile soil with a reduced capacity to support vegetation. It is estimated that water erosion affects 6.1 million hectares of cultivated soil in South Africa. Of this 15% is seriously affected, 37% moderately and the rest slightly. An estimated 10.9 million hectares of cultivated soil are affected by wind erosion. Of this 7% is seriously affected, 29% moderately and 64% slightly (DEAT, 1999).

5.2.4. Salinisation and sodification of soil

Salinity is primarily the result of low rainfall and declining water tables because of the increased exploitation of groundwater (Datta and De Jong, 2002). In arid and semi-arid regions leaching is not a dominant process and soluble salts accumulate in the upper part of the soil profile as a result of capillarity (Richards, 1994). Irrigation water, rain, wind-blown dust and processes of evaporation and transpiration also add to the concentration of soluble salts in soil. The accumulation of sodium salts leads to the more serious problem of

sodicity, increasing the amount of exchangeable sodium. This could lead to the swelling and dispersing of clay particles, causing deterioration in the soil structure (Rowell, 1994). Saline soil solutions take water away from plants causing dessication (Sanchez et al., 2003); while in sodic soils there is often a problem with water intake, transfer and aeration (Datta and De Jong, 2002). Most microorganisms are intolerant of high salt concentrations, and in saline soils they suffer from dehydration and the denaturation of proteins necessary for enzymatic activity (Atlas and Bartha, 1998). This threatens the sustainability of plant growth because of degradation in soil health and microbial activity (Datta and De Jong, 2002).

5.2.5. The effect of pH changes in soil

The soil pH is a very important factor in soil health. It regulates the solubility of nutrients and the bioavailability of potentially toxic heavy metals; controls the composition and diversity of the microbial community; alters the equilibrium solid phase and influences plant response (Atlas and Bartha, 1998; Riffaldi et al., 2002). It also affects soil enzymatic activity by influencing the concentration of inhibitors or activators in the soil solution and the effective concentration of the substrate. For example, increased pH results in higher alkaline phosphatase activity and lower acid phosphatase activity (Riffaldi et al., 2002). Natural soil processes, such as the biodegradation of plant material and mineral cycling activities (sulphur oxidation, nitrification), result in acid production (Atlas and Bartha, 1998). Another example of the significant effect of pH can be seen in the nitrogen cycle. Different microorganisms exhibit varying levels of sensitivity to pH. *Nitrobacter* spp. oxidise NO_2^- to NO_3^- and are more sensitive to high pH than *Nitrosomonas* spp., which oxidise NH_4^+ to NO_2^-. A shift in pH that places one group at a metabolic advantage can result in ammonia or nitrite toxicity (Richards, 1994).

5.2.6. The influence of soil pollution

Soils lose their ability to support plant growth when they become polluted (DEAT, 1999) and soil pollutants such as pesticides and heavy metals generally inhibit enzyme activity

because their presence places stress on the microbiota (Sannino and Gianfreda, 2001). In polluted soil, where microorganisms are under stress, the resilience/resistance of such a community is already compromised; therefore, any further stress would have a drastic effect on the structure and function of that microbial community (Majer et al., 2002).

Pesticides are one of the major anthropogenic factors that influence microbial function and structure in soil because of their continuous addition to the environment. Entry into soil ecosystems may be direct (e.g. agricultural application) or indirect (e.g. waste discharge, leaks at pesticide dumpsites, accidental spillage). The presence of pesticides, hydrocarbons and metals has variable effects on microbial enzymes. Some enzymes are inhibited by one pesticide, while the same pesticide activates another enzyme. An example of this is the herbicide glyphosphate that demonstrates activation of urease, but inhibition of phosphatase (Sannino and Gianfreda, 2001).

Heavy metals originate mainly from industrial processes such as mining activities and are toxic to organisms unless present in very low concentrations (Ashman and Puri, 2002). Wastewater being discharged into the soil environment (Tam, 1998); the use of wastewater for irrigation purposes (Ramirez-Fuentes et al., 2002); and the application of sewage sludge (Ashman and Puri, 2002) can also add to the levels of heavy metals present in soil. The presence of heavy metals in high concentrations in soil affects microbial activity (Kandeler et al., 1996; Majer et al., 2002). Metal contamination causes some enzymes, such as dehydrogenase, to be inhibited while other enzymatic activities, such as that of xylanase and protease increase in the presence of heavy metals (Majer et al., 2002). Other unfavourable consequences of heavy metal pollution include a negative effect on biomass carbon and nitrogen levels (Majer et al., 2002), and inhibition of denitrifying communities with an increase in atmospheric levels of dinitrous oxide (N_2O) (Holtan-Hartwig et al., 2002).

5.2.7. The influence of agricultural activities

Agricultural practices can have beneficial or detrimental consequences for soil quality. No-till management practices have been shown to maintain crop residue cover, organic carbon, and aggregate stability, thus improving the soil's resistance to erosion and ability to provide nutrients (Hussain et al., 1999). Excessive pest control, cultivation and unceasing monoculture cropping systems have led to unacceptable losses of already insufficient topsoil (Doran, 2002). Agricultural activities such as tillage and the addition of agrochemicals (pesticides, fertiliser) are likely to have an adverse effect on the composition of microbial communities in the soil. Tilled soil management systems have lower total organic carbon (TOC) and total nitrogen (TN) values, and all enzyme activities and biological indices exhibit lower values than that of untilled management systems (Aon et al., 2001). Lower metabolic activities occur with intensive cultivation compared to relatively undisturbed areas of a perennial nature (Rapport et al., 1997; Aon et al., 2001). Poor agricultural activities are responsible for the exploitation of soil nutrients, such as nitrogen, phosphorus and potassium, and can cause a critical decrease in soil fertility (Snakin et al., 1996). Pressure to increase productivity of agricultural systems to meet domestic and international demands has encouraged widespread use of fertilisers and other agrochemicals. Although the application of fertilisers is important to achieve optimum yields, it is equally important not to over-fertilise. Increased nitrogen inputs can change the composition of grassland vegetation and impact adversely on the soil microflora and nematode populations, with resulting changes in community structure (Sarathchandra et al., 2001). Over fertilisation increases the concentration of nitrates in underground and surface waters and of phosphates in surface waters. Excessive use of reduced sources of nitrogen (NH_4^+) is also a major contributor to soil acidification (DEAT, 1999). The presence of nitrate in drinking water can cause potentially fatal methemoglobinemia (blue baby syndrome) or even have carcinogenic effects when transformed in the body (Doran et al., 1996). When easily accessible fertiliser nutrients are available in the soil environment, the growth of r-strategic bacteria is promoted, consequently reducing the proportion of K-strategists (Sarathchandra et al., 2001). Changes like these are likely to be reflected in higher soil biota. In contrast to inorganic fertilisers, amendment of soil with organic

fertilisers increases the organic content of soil, which in turn leads to higher enzyme activities. Sufficient organic matter content is important to achieve sustainability in an ecosystem. Nevertheless, microbial activity will not increase above a soil's maximum organic matter content. The maximum will differ depending on the type of soil. Organic farming is being used increasingly in agriculture and can be distinguished from conventional agricultural practices by not making use of synthetic fertilisers and pesticides (Schjonning et al., 2002). A study by Glover et al. (2000), has illustrated the positive effects of organic farming on soil quality, such as increased aggregate stability, microbial biomass and earthworm abundance. Organic soil management practices include additions of composted poultry manure and bark mulches and the use of herbicides for weed control (Glover et al., 2000). However, the use of uncomposted organic residues has been shown to negatively affect crop yields and soil mycorrhizal populations (Caravaca et al., 2002).

5.2.8. The influence of mining activities

Mining in South Africa provides a vast contribution to the economy, both in terms of the actual materials that are mined and in the creation of literally hundreds of thousands of jobs, with benefits to all aspects of society (Mining Review Africa, 2003). Mining activities, however, inherently have extensive adverse effects on the biophysical, social and economic environment and results in severe disturbance of large land areas (Milton, 2001; Mummey et al., 2002a). The natural grassland biome of South Africa is fragmented by an abundance of mine tailings and discard sites, degrading the environmental quality of this poorly conserved biome and eventually affecting human living standards (Van Wyk, 2002). Tailings are being processed at a rate of millions of tons per year (Rösner et al., 2001) and there are approximately 400 massive tailings dams originating from coal, gold and base metal mining in South Africa (MJRS, 1996). In 1996, the mining industry was responsible for the production of 377 million tons of tailings, accounting for 81% of the solid waste stream in South Africa (Van Wyk, 2002). Currently, mining waste is still the biggest contributor to the solid waste stream, followed by pulverised fuel ash (6.7%), agricultural waste (6.1%), urban waste (4.5%) and sewage sludge (3.6%) (Arendse and Wilkinson, 2002). Cyanide compounds, heavy metals, radionuclides and asbestos are all possible

components of mine waste, often in the form of leachate. Water draining or leaching from mines can cause severe contamination of adjacent soils and water supplies, because it is often highly acidic and/or saline, with a high sediment load. If mining waste is not managed correctly, it represents a potential hazard for surrounding ecosystems and public health in nearby communities (Hoskin, 2003). Other impacts of mining include destruction of land and vegetation, pollution and changes in surface drainage. As a result, it can be expected that there will be an increase in soil erosion, compaction, subsidence and reduced capacity to support vegetation growth. Topsoil characteristics may also change with a possible increase in acidity and salinity, development of nutrient deficiencies or imbalances, surface crustiness or desiccation and changes in land use (Arendse and Wilkinson, 2002).

Although mines are expected to provide for and apply rehabilitating measures before closure is granted (Milton, 2001), it is much more complicated than simply restoring the disturbed area. The product of mining activities is a soil environment typified by poor physical characteristics, such as poor textural material properties, combined with the effect of the slopes of the discard sites (Van Wyk, 2002); low levels of plant nutrients and organic matter; pH extremes; and the presence of heavy metals (Mining Review Africa, 2003). The processing of mine tailings and discard material usually results in an elevated topography which means that these discard sites are particularly exposed to the adverse effects of wind and water erosion (Van Wyk, 2002). These aspects, often accompanied by difficult climatic conditions characteristic to arid and semi-arid areas of southern Africa, deter the establishment of permanent self-sustaining vegetation cover (revegetation) on mine stockpiles and tailings (Milton, 2001; Mining Review Africa, 2003). Past rehabilitative management strategies that relied on the restoration of disturbed areas by botanical means proved unsuccessful (Van Wyk, 2002). However, it seems that vegetative stabilisation is the most successful answer to achieving sustainable rehabilitation of mine discard sites (Carroll et al., 2000) and since soil is the growth medium for all vegetation, it is important to find suitable methods to assess and improve the quality of this growth medium. Research should thus be focused on a more integrated approach that takes physical, chemical and biological properties and their interactions into account.

6. ASSESSING ECOSYSTEM HEALTH

6.1. Towards Rehabilitation and Sustainability

The Society for Ecological Restoration defines ecological restoration as "the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (SER, 2002). Along with water, soil is the most vital natural resource available to man, making its preservation and the restoration of already damaged environments critical in achieving sustainable development and feeding the growing world population (Arshad and Martin, 2002). It is fundamental in both the functioning of ecosystems and as an economic resource required for agriculture, silviculture, supply of raw materials and as a platform for infrastructural development (Rapport et al., 1997).

The extent of anthropogenic disturbance to terrestrial ecosystems has stimulated research to evaluate the impact of these stresses on ecosystem biota. The reason for this being that it is now realised that evaluation is the first step to ecological restoration and that it is of the essence to our survival to repair degraded ecosystems (Hobbs and Harris, 2001) because soil, unlike water is non-renewable on a human time-scale (Doran and Safley, 1997). Human-induced influences on soil change its properties, altering the soils ability to sustain equilibrium in the environment. It is therefore vital that these properties should be measured and the measurements understood in order for discussions concerning effective management or environmental issues to be founded on exact information (Rowell, 1994). The ultimate goal of all rehabilitation decisions should be to establish restoration in disturbed ecosystems to such a degree that the ecosystem can reach a state of sustainable equilibrium (stability). Karlen et al. (2003) illustrated the relationship of environmental quality to economic sustainability and social viability (Figure 3). Sustainable soil ecosystems that are able to support the needs of growing populations can only be attained if the necessary attention is paid to all of these interrelated aspects.

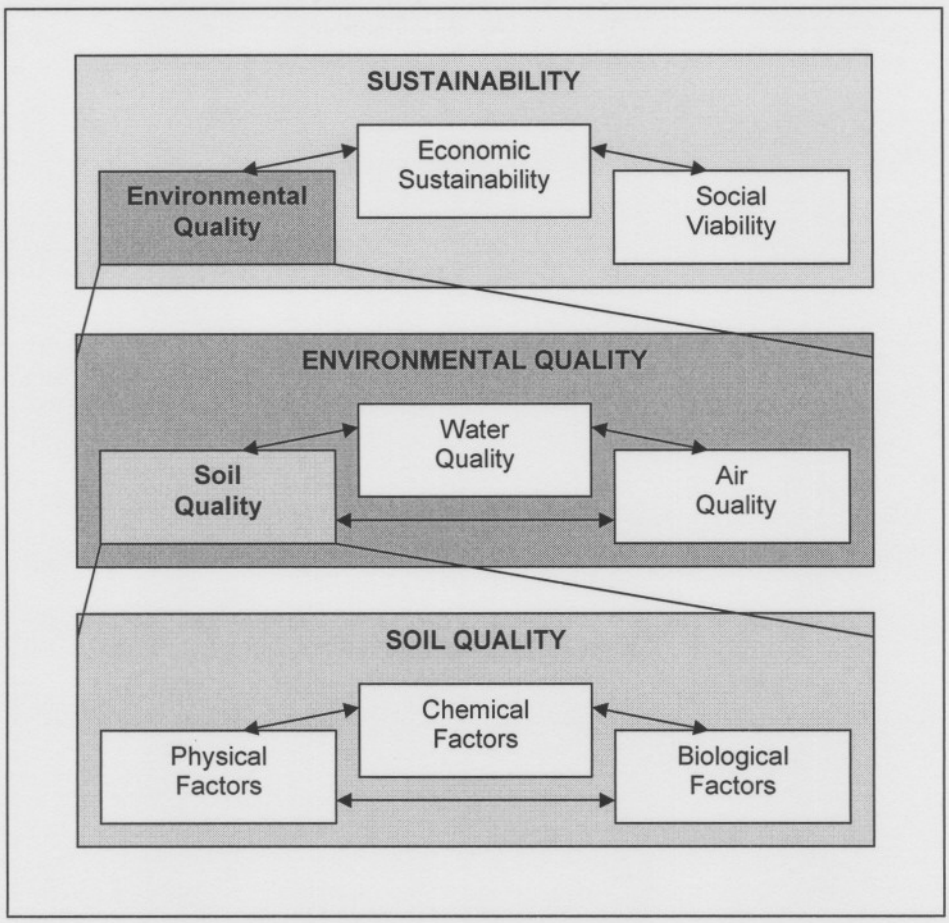

Figure 3. Hierarchical relationship of soil quality to sustainability (adapted from Karlen et al., 2003).

6.2. Methods to Assess Soil Quality

Soil that is 'healthy' or has a high 'quality' implies that the soil maintains a certain normality of function over a period and is free of symptoms of 'disease'. Considering this and the multitude of processes that play a role in overall ecosystem health, it is clear that soil quality cannot be measured directly. Instead, it should be assessed by looking at key components or processes of the soil ecosystem that might signify malfunction (Elliot, 1997). The monitoring of soil quality relies on the use of indicators. The application of indicators is practical because it provides a simplified mechanism for acquiring feedback on system changes and consequently improves the chances of making informed

management decisions (DEAT, 1999). In the case of soil quality, an indicator should be a measurable soil attribute that determines how well a soil functions (Schoenholtz et al., 2000), or if a specific management strategy has a positive or negative influence on soil quality. The selection of suitable indicators is complicated for a number of reasons. It is important to realise that results obtained from the individual measurements of soil ecosystem components represent the summed response of the whole system (Elliot, 1997). Characteristics used to monitor the health of a soil ecosystem should be those that respond to changes (including management practices) over the medium term because they integrate the effects of different soil processes and management activities over time (Rapport et al., 1997). A myriad of physical, chemical and biological properties determine the overall health of a soil ecosystem. It is therefore essential that potential ecological indicators should be physical, chemical or biological elements of ecosystem structure or function (Van Wyk, 2002). In order to achieve a comprehensive assessment of soil quality it is necessary to take into account both the numerous dimensions of soil function, such as productivity and environmental fitness, and the assortment of physical, chemical, and biological factors which control biogeochemical processes; and their variation in intensity over time and space (Doran and Safley, 1997).

Traditionally, physical and chemical analyses of soil, such as pH, water holding capacity, and total carbon and nitrogen contents have been used in the characterisation of soil. Therefore, these properties formed the foundation for the majority of management decisions. Chemical and physical analyses are, however, slow to change and the response of these parameters can only be measured effectively over an extensive period (Pascual et al., 2000). Furthermore, soil is a very complex ecosystem and the analysis of physical and chemical characteristics does not allow conclusions to be made concerning the biological structures and functions within the soils (White et al., 1996; Widmer et al., 2001). It is, nevertheless, important that qualitative information be included when monitoring soil quality. The occurrence of certain morphological phenomena, such as loss of organic matter, water and wind erosion, salinisation, acidification, and poor drainage and structural deterioration are important signs of degradation in soil quality (Doran and Safley, 1997).

The significance of microbially-driven soil processes in mediating global climatic change, by acting as carbon sources and sinks, and generation of greenhouse gasses such as nitrogen oxides and methane, makes them a logical choice as potential indicators of ecosystem health (Riffaldi et al., 2002; Ritz et al., 2003). Subsequently, microbiological and biochemical properties of soil have often been proposed as early and sensitive indicators of soil ecological stress or restoration processes because they are responsive to small changes that occur in soil (Bandick and Dick, 1999; Badiane et al., 2001; Ibekwe et al., 2002). Previously, the analysis of soil microbial communities has relied extensively on culture dependent techniques using a variety of enriched culture media. These conventional microbiological techniques are, however, insensitive and provide little insight into the nutritional and/or environmental status *in situ* (Hill et al., 2000).

Soil properties used as indicators of soil health or soil quality should adhere to certain criteria in order to be efficient. These criteria pertain primarily to the indicators' efficacy in defining ecosystem processes and integrating physical, chemical and biological properties (Doran and Zeiss, 2000). Certain important criteria for the use of soil microorganisms as indicators of soil quality are discussed as set out by Doran and Zeiss (2000). Firstly, an indicator should be sensitive to long-term variations in management, but not so sensitive as to be influenced by short-term weather patterns. Secondly, it should be well correlated with beneficial soil functions such as decomposition. The third criterion to be met is whether an indicator is useful for elucidating why ecosystem processes function well or not, not merely for predicting if a soil will provide beneficial function. Fourthly, indicators should be comprehensible and useful to land managers and as easy and inexpensive to measure as possible. Soil microorganisms meet the first three criteria because they respond sensitively to anthropogenic disturbance, their diversity is well correlated with many beneficial soil functions and they play a direct role in many ecosystem processes. However, more research is needed to develop sampling protocols compatible with time constraints imposed on land managers. It remains to be seen whether these potentially valuable biological indicators could be made available to land managers (Doran and Zeiss, 2000) The development of assay kits that include a comprehensive set of biosensors may make the assessment and

management of soil quality more accessible. Assay kits for the estimation of enzymatic activities are currently being tested (Vepsäläinen et al., 2001).

6.2.1. Conventional microbiological techniques and associated problems

The classification and identification of microorganisms based on morphological traits is complicated because microorganisms are small and lack conspicuous external features (Muyzer, 1999). According to Bengtsson (1998), high measurements of biodiversity have been correlated with high soil quality in past studies; however, diversity and ecosystem function are not directly linked, but can rather be correlated through the interactions of functional groups. It would therefore be more meaningful to focus on functional groups and species whose interactions bring about ecosystem function (Bengtsson, 1998). The analysis of soil microbial communities has relied primarily on culturing techniques using a variety of enriched culture media (Hill et al., 2000).

Conventional microbiological techniques, which are dependent on the culturability of the microorganisms being studied, are often insensitive and provide little insight into the nutritional/environmental status *in situ* (White et al., 1996). Standard plate count methods may only recover a small percentage of the organisms present (Peacock, et al., 2001). There are two reasons for this. In the first place, it is difficult to extract microorganisms from soil. Even after multi-stage extractions using chemical and physical dispersion treatments, large proportions of microorganisms remain associated with soil particles. Another reason for inaccurate estimation of numbers is that the isolated microbes are restricted to those that can grow on the medium of choice (Peacock et al., 2001; Taylor et al., 2002). According to Hill et al. (2000), it has been estimated that less than 0.1% of the microorganisms found in typical soil environments are culturable using current culture media formulations. This can be attributed to the ignorance concerning culture conditions under which microbes thrive in their natural environment (Muyzer, 1999). Culturable microbes recovered from environmental samples thus represent only a fraction of the extant microbiota (White et al., 1996).

The traditional measurement of non-viable microbial biomass by the chloroform fumigation-extraction (CFE) or chloroform fumigation-incubation (CFI) methods is also used to study microbial communities (Bailey et al., 2002). The use of CFE or CFI provides a direct measurement of total soil biomass (Wang et al., 2003) and has the advantage of not requiring direct counts and size conversions (Elliot, 1997). These methods give an indication of the function of microbial life as a pool, but provide no information on community structure (Alef and Nannipieri, 1995; Peacock et al., 2001). Other negative aspects include different extraction efficiencies for different soils and difficulties in separating root from microbial biomass (Elliot, 1997).

6.2.2. Alternative approaches

Numerous alternative techniques, which circumvent the problems frequently associated with conventional microbiological techniques, have subsequently been proposed. These recently developed techniques provide a more detailed investigation of soil biological parameters (Widmer et al., 2001). These methods include analyses of nucleic acids (Muyzer, 1999); community level substrate utilisation (CLSU) profiles or community level physiological profiles (CLPP) (Hill et al., 2000); the estimation of enzymatic activities (Garcia et al., 2002); and signature lipid biomarker analyses (Ibekwe et al., 2002). Profiles of substrate utilisation and enzyme activity represent the functional diversity, whereas nucleic acids and signature lipid profiles reflect the structural composition of microbial communities (Hill et al., 2000).

6.2.2.1. Molecular analyses

Genetic analysis relates to the fundamental information or base structure of the microbial community that is present (Ritz et al., 2003). A number of the difficulties associated with the study of microorganisms *in situ* can be overcome by studying them at the genetic level. Molecular techniques, which are culture-independent, have the advantage over analyses of substrate utilisation and enzymatic activity of not being limited to substrate reactions and they provide a better understanding of the processes mediated by microorganisms (Peacock

et al., 2001). Of all the cell component molecules tested to date, nucleic acids have been the most useful in providing a new understanding of the structure of microbial communities. Research has shown that the genetic diversity of a studied soil was 200 times greater than the diversity among bacteria cultured from the same soil. This indicates that soil microbial communities are much more complex than is currently recognised, and that the analysis of DNA sequences may provide a greater understanding of the microbial diversity that exists in soil than could be gained from culture-dependent methods (Hill et al., 2000).

A number of molecular techniques are available to investigate microbial community structure. The majority of molecular techniques rely on the isolation and amplification of specific nucleic acid sequences, with extraction from molecular material being either direct or indirect. Where direct extraction is applied, previously undescribed microorganisms are included in the procedure (Ritz et al., 2003). Hybridisation techniques that make use of specific gene probes may be used to track population dynamics and shifts in dominance (Muyzer, 1999; Ritz et al., 2003). The use of ribosomal ribonucleic acid (rRNA) to analyse microbial communities is functional in identifying community members with high specificity to species and strain level and can detect phylogenetic affinities of uncultured organisms. The use of the 16S rRNA gene as an indicator of species diversity is a more recent approach for investigating the structure of microbial communities (Ibekwe et al., 2002). It is also possible to establish phylogenetic relationships between microorganisms in a sample and to compare communities from different habitats (Ritz et al., 2003). Denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) as genetic fingerprinting techniques for separating individual amplicons and studying the successional population changes in microbial communities has recently been described (Muyzer, 1999; Hill et al., 2000).

Genetic fingerprinting can provide a direct diversity profile of complex microbial communities based on the physical separation of unique nucleic acid species (Muyzer, 1999). The use of DGGE or TGGE is based on electrophoresis of polymerase chain reaction (PCR) -amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (Muyzer et al., 1993). When using DGGE, the

denaturing agent is chemical (urea or formamide); on the other hand, the denaturing agent for TGGE is a physical factor, such as temperature (Ranjard et al., 2000). Separation of deoxyribonucleic acid (DNA) fragments is based on the electrophoretic mobility of partially melted DNA molecules in polyacrylamide gels containing a linear gradient of a DNA denaturing agent. The electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gel is decreased compared with that of the helical form of the molecule (Muyzer et al., 1993). Different DGGE bands separate according to the melting behaviour of PCR products and not the size of the nucleotide fragment (Moesender et al., 1999). Molecules with different sequences exhibit different melting behaviour, resulting in the molecules stopping their migration at different positions in the gel (Muyzer, 1999). The denaturation of DNA takes place in 'melting domains' (Muyzer et al., 1993); these are stretches of base pairs with an identical melting temperature. A molecule stops migrating when a transition takes place of helical to partially melted molecules. This occurs once the melting domain with the lowest melting temperature reaches that position on the DGGE gel where there is a corresponding melting temperature (Muyzer et al., 1993).

Denaturing gradient gel electrophoresis allows for the monitoring of complex microbial community dynamics due to seasonal fluctuations or after environmental perturbations because multiple samples can be analysed simultaneously (Muyzer, 1999). It has also made identification of the presence and relative abundance of different species possible, thus providing a qualitative and semi-quantitative manner of profiling microbial populations (Muyzer et al., 1993). According to Muyzer (1999), DGGE and TGGE are used worldwide in many environmental microbiology studies as a routine molecular tool for comparing the diversity of microbial communities and for the monitoring of microbial population dynamics. The use of DGGE has been applied for the fingerprinting of natural bacterial populations (Muyzer et al., 1993) both in terms of the total bacterial community or particular populations (Muyzer, 1999); the study of community diversity in soil (Ranjard et al., 2000) and sediments (Spring et al., 2000); and the characterisation of microbial communities in water (Fonseca et al., 2001) and hydrothermal vents (Brinkhoff et al., 1999). Muyzer et al. (1993) demonstrated that DGGE is sensitive enough to identify constituents that represent only 1% of the total population. This technique also has the

capacity to identify community members by the sequencing of excised bands or by hybridisation analysis with specific probes and is rapid, reproducible, reliable and inexpensive (Muyzer, 1999). Genetic analysis makes the studying of unculturable microorganisms attainable (Zhou et al., 1996) and holds the considerable advantage that microorganisms from natural habitats can be studied and characterised without culturing. Over 90% of the microorganisms *in situ* can be extracted and analysed using molecular techniques, as compared to the less than 0.1% that can be recovered from culture media (Hill et al., 2000).

Despite the usefulness of genetic analyses, there are a number of limitations. Ritz et al. (2003) stated that molecular approaches applied to DNA holds the disadvantage that most of the rapidly captured data reflects the total, not the expressed diversity. Other problems involve the storage of samples prior to processing; differences in extraction efficiency among soils and microorganisms; variation in the affinity of microorganisms for probes; and incomplete lysis of some prokaryotic cells. All of these factors could result in biased estimates of activity (Hill et al., 2000). Another important limitation is that eukaryotic ribosomes, such as that of fungi, have not been well studied because the approach has been applied mostly to investigations of prokaryotes (Muyzer, 1999; Hill et al., 2000).

6.2.2.2. Functional analyses

The use of functional profiles to gauge biological status is a valuable tool, since it relates to the actual or potential activities of organisms that ultimately result in ecosystem dynamics. The biogeochemical cycling of nutrients, such as carbon, nitrogen, and phosphorus is a fundamental soil function and therefore of great interest in terms of assessing the relative activity of soil microbial communities (Ritz et al, 2003). In this context, CLSU profiles and assays of the enzymatic activities of microorganisms are often used to determine the functional diversity of microbial communities. In both types of analyses, the ability of the microbial population to utilise a specific substrate is measured.

6.2.2.2.1 Community level substrate utilisation profiles

Community level substrate utilisation (CLSU) profiles, also referred to as community level physiological profiles (CLPP) or metabolic fingerprinting (Hill et al., 2000; Widmer et al., 2001), is one such method of functional analysis. An example of a CLSU method is the Biolog® system. This system was initially developed for the identification of pure bacterial strains, but it is widely used in environmental studies to characterise microbial communities based on the statistical analysis of their carbon source utilisation patterns (Guckert et al., 1996). One of the first demonstrations of the characterisation of diversity in microbial community function between different habitats and different samples within the same habitat based on CLSU profiles, was made by Garland and Mills (1991).

Biolog® microtiter plates (Biolog Inc., Hayward, USA) are commercially available for both Gram-negative and Gram-positive microbes and the system is based on the utilisation of a suite of different carbon sources. Each plate contains 95 wells with pre-dried carbon sources and a tetrazolium violet redox dye (Guckert et al., 1996), and selects for microorganisms that metabolise under the given conditions on the microtiter plate (Palojarvi et al., 1997). Each carbon-compound acts as a carbon, energy and electron source for the microorganisms (Lowit et al., 2000). When microorganisms utilise the carbon substrate, the tetrazolium violet turns purple and this colour development is then quantified spectrophotometrically. The substrate utilisation profile data is analysed using multivariate statistical techniques and a reflection of the metabolic capabilities of a part of the community is obtained (Garland and Mills, 1991; Guckert et al., 1996).

Compared to community level approaches based on, for example, DNA analysis, the Biolog® system is a relatively simple and rapid technique (Buyer and Drinkwater, 1997). It also '...has the capacity to produce a rich data set that is ideal for detecting site-specific differences in the functional diversity of soil bacteria and for evaluating the relationship between biodiversity and the expression of function in a natural ecosystem' (Pankhurst, 1997). This assay has been widely used to describe the functional diversity of microbial communities in various soil and water environments under different management strategies (Guckert et al., 1996; Kelly and Tate, 1998; Smalla et al., 1998).

There are, however, several limitations to the Biolog® system. Firstly, false negatives can occur because of a substantial lag phase during cell growth and the period of microbial growth within the well. A second problem is that analysis of functional diversity is based on the assumption that colour development in each well is solely a function of the proportion of organisms present in the sample which are able to utilise a particular substrate. This can be invalid because some strains may predominate in certain wells because they exploit specific substrates more efficiently than others do. Thirdly, substrates in commercially available Biolog® plates do not necessarily reflect the diversity of substrates found in the environment (Hill et al., 2000). Only a few percent of the soil microorganisms present in a soil sample are capable to grow and metabolise under the *in vitro* conditions of the Biolog® method and as a result, the greater part of the soil community is not represented (Ritz et al., 2003). It is also important to realise that it cannot be assumed that an organism will utilise a specific compound as a substrate in soil just because it utilised the substance under laboratory conditions (Richards, 1994). A further disadvantage, according to Haack et al. (1995), is that a minimum number of metabolically active cells ($\approx 10^8$ / ml) are required to produce an observable colour change. Even though the Biolog® method offers a limited window upon the probable substrate-utilisation capacity of soil communities, it demonstrates the basic concept of catabolic fingerprinting as acceptable (Ritz et al., 2003). The Biolog® assay, although also culture-dependent, provides more reproducible results than conventional culturing methods. This is especially significant when evaluating changes in the metabolic diversity of mixed microbial communities. Nevertheless, culture-based techniques should not be used alone to study the composition of microbial communities. They are more practical when used in combination with techniques that are culture-independent (Guckert et al., 1996; Hill et al., 2000).

6.2.2.2.2 Substrate Induced Respiration

Substrate induced respiration (SIR) was first introduced by Anderson and Domsch (1978) for the measurement of soil microbial biomass. Soil microorganisms are often dormant and have low respiration rates. The substrate induced respiration (SIR) method stimulates respiration through the addition of a readily decomposable substrate, such as glucose (Lin

and Brookes, 1999). The measured response of the soil microorganisms induced by glucose can then be proportionally related to the size of the original microbial biomass (Bailey et al., 2002). This method can be modified to selectively inhibit bacterial and fungal respiration by the addition of population-specific antibiotics. Bacterial respiration is usually inhibited by streptomycin and eukaryotic respiration, such as that of fungi, by cycloheximide. In this way, it is possible to quantitatively determine the individual contributions of the bacterial and fungal populations, respectively, to respiration in different types of soil (Lin and Brookes, 1999; Tate, 2000).

Some limitations to the selective inhibition of the SIR method are of importance. The antibiotics used are often insufficiently specific and may be inactivated or degraded by surviving soil microorganisms (Lin and Brookes, 1999). Antibiotics sorbed onto organic matter or clay surfaces are also inactivated (Tate, 2000). The effective concentration of antibiotics may thus be significantly lower than the total quantity added, and this should be considered in accordance with the type of soil under investigation (Tate, 2000). The use of selective inhibitors suppresses the metabolic activities of bacterial and fungal populations. However, this may result in additional energy becoming available to organisms of which metabolism is not suppressed (Lin and Brookes, 1999).

6.2.2.2.3 Estimation of enzymatic activities

The presence of enzymes is vital for all biochemical transformations in soil, thus, the study of soil enzymatic activities provides insight into microbial dynamics and populations (Riffaldi et al., 2002). Enzyme sources in soils include plant, animal, fungal and bacterial cells. Table 6 shows the major groupings of commonly assayed soil enzymes and their ecological function.

Table 6. Major groupings of commonly assayed soil enzymes and their ecological function (Dick, 1997).

EC[1] group and subgroup number	Enzyme	Ecological or soil health function
Oxidoreductases		
1.1	Dehydrogenase	Exists as integral part of intact cell and reflects total oxidative activities of soil microflora; important in oxidising soil organic matter
1.1	Glucose oxidase	Oxidises glucose
1.11	Catalase, peroxidase	Release oxygen from hydrogen peroxide
1.10	Polyphenol oxidases	Oxidise phenolic compounds and are involved in humification of soils
Hydrolases		
3.1	Phosphatase (mono- and diester)	Releases plant available PO_4 from organic matter
	Sulphatase	Releases plant available SO_4 from organic matter
3.2	Amylase	Hydrolyses starch into maltose
	Cellulase	Endohydrolysis of 1,4-β-D-glucosidic linkage in cellulose, a major component of wood and plant fibres
	Xylanase	Cleaves 1,3-β-D-xylosidic linkages of xylan, a polysaccharide found with cellulose
	β- and α-glucosidases	Release glucose, an important energy source for microbial activity
	β- and α-glucosidases	Hydrolysis of melibiose and lactose, respectively
	Invertases, saccharase, sucrase	Hydrolyses sucrose to glucose and fructose, providing energy for microbial activity
3.4	Proteinases	Hydrolyses proteins, releasing amino compounds; important in N cycle and N mineralisation
	Peptidase	Hydrolyses dipeptides, releasing 2 amino acids; important in N cycle and N mineralisation
	Amidase	Hydrolyses C-N bonds of amides releasing NH_3; important for N mineralisation to provide plant available N
	Urease	Belongs to group of enzymes acting on C-N bonds of urea, a fertiliser source and a major constituent in urine of grazing animals
3.5	Arylacylamidase	Hydrolyses propanil, which is used as a herbicide
Transferases		
2.4	Dextransucrase	Hydrolyses sucrose, releasing glucose and fructose
2.8	Thiosulphate *S*-transferase (rhodanese)	Performs intermediate step in oxidation of elemental S which is found in small amounts in soils or is added as a S fertiliser
Lyases		
4.1	Glutamate decarboxylase	Hydrolyses aspartic acid
4.1	Tyrosine decarboxylase	Hydrolyses tyrosine, a product of proteinase activities and involved in N mineralisation
4.3	L-Histidine ammonia lyase	Deaminates histidine and involved in N mineralisation
Broad spectrum enzyme assay	Fluorescein diacetate hydrolysis	Provides general indication of soil hydrolytic activity by assaying 3',3'-diacetylfluorescein hydrolysis which is carried out by proteases, lipases, and esterases

[1]Enzyme Commission

The measurement of a variety of enzymes in soil gives an indication of the diversity of functions that can be assumed by the microbial community (Brohon et al., 2001). Studies have shown that enzyme activity such as that of dehydrogenase, β-glucosidase, urease and phosphatase show significant correlation with total organic carbon, total nitrogen, water-filled pore space (WFPS), and heterotrophic bacterial and fungal biomass (Aon and Colaneri, 2001). It is however, of great importance to realise that not all enzymatic activities are directly related to microbial activity. For example, a reaction may be catalysed in a soil microsite yet provide no metabolic benefit for the soil biological community because the products of such a reaction are unavailable to the cells (Tate, 2000). Different types of enzymatic activities can be distinguished. Cells contain intracellular enzymes and are the primary source of extracellular enzymes. Intracellular enzymatic activities are involved in cellular metabolism and cannot function outside the cell due to their sensitivity to environmental factors. In contrast, extracellular enzymes are secreted by living cells and their activity is directed towards external functions. Constitutive enzymes are continuously produced by the cells, while others are only produced when their activities are required (inducible) (Tate, 2000). Some enzyme activities in soil can be associated with exoenzymes released from active cells (animal, plant, microbial), endoenzymes released from disintegrated cells, as well as being complexed with clay minerals and humic colloids (humified) (Kiss et al., 1975; Aon and Colaneri, 2001; Taylor et al., 2002). Humified enzymes assume degradation properties similar to that of the humic acid – for example years, decades or even centuries. This means that an enzyme can survive long after the cell that produced it, in other words, it has been separated from the living world and has become part of the non-living world; such enzymes are termed abiontic (Tate, 2000).

Enzyme assays are process level indicators and a culture-independent method (Dick, 1994). The activity of any enzyme assayed in a soil sample is the sum of active and potentially active enzymes from all the different sources (Tate, 2000). Therefore, results are presented as a means of determining the potential of a soil to degrade or transform substrates (Dick, 1994). What further complicates the interpretation of enzymological data is the fact that not all enzymes catalysing a specific reaction are assayed under their optimal conditions (Tate,

2000). Soil enzymes have been suggested as potential indicators of soil quality because of their essential role in soil biology, ease of measurement and rapid response to changes in soil management (Bandick and Dick, 1999; Riffaldi et al., 2002; Turner et al., 2002).

Soil enzymes representative of the main biogeochemical cycles (C, N, P, S) and of microbial biomass are often used as indicators of soil quality (Table 6). Enzymes often studied in this context include β-glucosidase (EC 3.2.1.21) (C cycle), urease (urea amidohydrolase, EC 3.5.1.5) (N cycle), and acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase (P cycle). Dehydrogenase activity is measured as an estimation of overall microbial activity (Bandick and Dick, 1999; Masciandaro and Ceccanti, 1999). Dehydrogenase is present in all microorganisms and assays are considered an accurate measure of the overall microbial activity in the soil with a direct relationship to total viable microorganisms (Taylor et al., 2002). Studies have shown (Garcia et al., 2002) that the type of substrate can influence the dehydrogenase activity, with lower values being observed in acid soils. Enzymatic activity in the soil environment is a major contributing factor to overall soil microbial activity (Garcia et al., 2002). β-Glucosidase, urease, and acid and alkaline phosphatases are enzymes that carry out specific hydrolyses and catalyse reactions involved in the biogeochemical transformations of carbon, nitrogen, and phosphorus, respectively (Aon and Colaneri, 2001; Taylor et al., 2002). β-Glucosidase activity is very useful in the monitoring of soil ecosystems for several reasons. It plays a central role in the cycling of organic matter, is the most abundant of the three enzymes involved in cellulose degradation and is rarely substrate limited (Turner et al., 2002). Urease and phosphatase activities are often measured because of their importance in the N and P biogeochemical cycles (Sannino and Gianfreda, 2001). Enzymatic activities in relation to the cycling of nitrogen (ammonification, nitrification, denitrification) or phosphorus (release of inorganic phosphorus) in soil have been used to evaluate the fertility of the soil or to describe the functioning of the ecosystem (Brohon et al., 2001; Aon and Colaneri, 2001). Arylsulphatase (EC 3.1.6.1) plays an important role in the cycling of sulphur and has been studied for its relationship to microbial activity. This enzyme can catalyse the hydrolysis of

organic sulphate esters and can sometimes constitute a rate-limiting stage in the cycling process. Bacteria and fungi are the main origins of arylsulphatase, although plants and animals also produce this enzyme (Li and Sarah, 2003).

One of the disadvantages of enzymatic measurements is the possible adaptation of the microorganisms in contaminated soils, which could lead to biased results (Brohon et al., 2001). Enzymes appear to have limitations specifically with respect to their ability to reflect pollution degradation. In particular, the measurement of enzyme activity does not distinguish between degradation due to pollution and prior degradation of the soil at the pollution site, which in turn hinders comparison of observations made at different sites (Trasar-Cepeda et al., 2000). It is also necessary to take spatial and temporal variation of enzymatic activities into account when assessing soil quality based on these assays. Enzymatic activity decreases with depth of soil, with the highest activity in the surface soil layer (0-8 cm) (Aon and Colaneri, 2001), and should furthermore, be monitored as trends over time. This would eliminate problems with seasonal fluctuations and inherent differences in activity (Bandick and Dick, 1999).

6.2.2.3. Phenotypic analyses

According to Ritz et al. (2003), the phenotypic analysis of a microbial community '...relates to the prevailing expression of the genetic background, i.e. the 'living form' of the community'. The use of a culture-independent method (Hill et al., 2000), such as phospholipid fatty acid (PLFA) analysis is a powerful means to examine microbial community structure. This technique is an example of signature biomarker analysis and circumvents many of the problems frequently associated with conventional culture-dependent techniques. It also provides a more comprehensive view of the structure of complex microbial communities than the conventional techniques (Pinkart et al., 1998; Waldrop et al., 2000). Phospholipid fatty acid profiles are composed of fatty acids of varying chain length, saturation and branching and can therefore be used as 'fingerprints' of the soil community (Steer and Harris, 2000; Bailey et al., 2002).

The analysis of PLFAs is based on the extraction and fractionation of the microbial lipid extract into neutral lipids, glycolipids and phospholipids by silicic acid chromatography. The lipid fractions are then quantitatively analysed using capillary gas chromatography and gas chromatography-mass spectrometry (Guckert et al., 1985; White and Ringelberg, 1998). The glycolipids and phospholipids are part of the polar lipid fraction that has a polar head group. The neutral lipids, on the other hand, contain no charged atoms (Kock and Ratledge, 1993).

Phospholipid fatty acids are present in the cell membranes (White et al., 1996) of all living microorganisms and function to maintain cell fluidity, enable transport of nutrients into the cell and eliminate metabolic products (Ponder and Tadros, 2002). Since PLFAs are not associated with storage functions, they represent a constant portion of the cell mass (Noble et al., 2000). Following cell death, PLFAs are rapidly degraded by endogenous and exogenous phospholipases (Peacock et al., 2001), which makes them valuable as signature molecules and indicators of viable microbial biomass (Calderón, 2000; Rütters et al., 2002). Viable biomass can be measured either as lipid phosphate (Guezennec and Fialu-Medioni, 1996) or as ester-linked fatty acids (White et al., 1996). In addition, the physiological status and community structure can be inferred from lipid profiles (Steenwerth et al., 2003) since certain fatty acids are unique to specific groups of organisms (Table 7). Phospholipid fatty acid profiles can therefore signify changes in the microbial composition of a soil (Ibekwe and Kennedy, 1998; Hill et al., 2000).

Table 7. Phospholipid fatty acids (PLFAs) community structure groups associated with the membranes of various microorganisms (Olsson, 1999; Ponder and Tadros, 2002; Rütters et al., 2002; Steger et al., 2003).

PLFA structure group	General classification
Normal saturated	A general microbial biomarker found in both the prokaryotic and eukaryotic (polyenoic fatty acids) kingdoms; a relative increase has been shown to correlate with decreased diversity.
Terminally-branched saturated	Representative of Gram-positive bacteria, including *Arthrobacter* and *Bacillus* spp. Many of these types of bacteria can be spore formers and can exist in environments that are lower in overall organic carbon content.
Mid-chain branched saturated	Primarily indicative of Actinomycete type bacteria in surface soils. It has been hypothesised that since these bacteria grow hyphae they are able to better survive in harsh environments due to their ability to span interstitial spaces to collect water and nutrient sources.
Monounsaturated	Indicative of predominantly Gram-negative bacteria, which is fast-growing, utilise many carbon sources and adapt quickly to a variety of environments; may also be found in the cell membranes of obligate anaerobes such as sulphate or iron-reducing bacteria; an increase in the amount and type of carbon sources has been shown to increase this marker.
Polyunsaturated	Representative of fungi and other microeukaryotic organisms; this marker too shows significant differences due to land-use.

The composition of PLFAs in microorganisms is affected by the metabolic state of the organism, environmental factors and exposure to toxic substances (Frostegard et al., 1997). Accordingly, when bacteria are cultured under standardised conditions, they maintain a constant fatty acid composition unique to specific groups of microorganisms (Keweloh and Heipieper, 1996). Another advantage of this specific procedure of PLFA analysis is that PLFAs are easily extracted from soil and the technique is optimised for phospholipid molecules, so that other free fatty acids are not detected. Consequently, it provides insight into a greater portion of the whole community composition than culture-dependent practices would (Hill et al., 2000; Peacock et al., 2001).

The use of PLFA analysis to differentiate between bacterial and fungal biomass, is very useful, since other techniques, such as SIR and direct microscopy is time-consuming and sometimes imprecise (Bååth and Anderson, 2003). Frostegård and Bååth (1996) suggested the use of the sum of PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy 17:0, 18:1ω7 and cy 19:0) as an index of the bacterial biomass (BactPLFA). The quantity of 18:2ω6 was used as an indicator of fungal biomass (FungPLFA), since it is mainly of fungal origin (Merilä et al., 2002). According to Bailey et al. (2002), the fungal/bacterial (F:B) ratios obtained from PLFA analysis are similar to those obtained by selective inhibition of substrate-induced respiration, with a F:B activity ratio of 1.0 indicating equal contribution of fungi and bacteria to the microbiological activity in the soil sample. When using PLFA analysis to determine F:B ratios, the ratio is usually less than 1.0 since the saturated fatty acids included in the prokaryotic lipids are ubiquitous and found in most organisms (Bailey, et al., 2002). In a study conducted by Bardgett and McAlister (1999), F:B ratios were found to be indicative of ecosystem self-regulation. Results from this study suggest that the ratio of F:B PLFA is higher in soils that are unimproved, in contrast to soils that have been fertilised and show a lower ratio of F:B PLFA. The ratio of F:B biomass was also positively correlated to soil pH. An increase in F:B PLFA was measured with increasing soil pH (Bååth and Anderson, 2003). Similarly, native soil systems show a tendency to be characterised by high F:B ratios compared to managed systems (Bardgett and McAlister, 1999). Following this, Zeller et al. (2001) has shown the absolute fungal biomass to be more sensitive than the total soil microbial biomass to detect the effect of management abandonment in grassland soils. This is of great value when comparing the long-term effects of management or environmental changes on otherwise similar sites (Bailey et al., 2002).

The ratio of diglyceride fatty acids (DGFAs) to PLFAs provides an estimate of the ratio of non-viable to viable microbial biomass (Dowling et al., 1986). Patterns of DGFAs could indicate recently lysed components of the microbial community (White, 1995) because they are formed when cellular enzymes (phospholipases) hydrolyse the phosphate group of the phospholipid (Kieft et al., 1994).

The growth rate, medium composition and environmental factors under which microorganisms grow, influence the relative amounts of *trans* fatty acids present in the cells. Accordingly, the measurement of the *trans/cis* isomeration of fatty acids can be used to determine the physiological status of the microbial population (Keweloh and Heipieper, 1996). Mandelbaum et al. (1997) suggested the use of the *trans/cis* ratio of 16:1 fatty acids and 18:1 fatty acids as a general measure of stress or starvation. The concentration of *trans* monoenoic fatty acids usually increases during nutrient deprivation, while the concentration of *cis* monoenoic fatty acids decline (Guckert et al., 1986). *Trans/cis* ratios greater than 0.1 are considered indicative of starvation or exposure to toxins (Keweloh and Heipieper, 1996; White et al., 1996). In contrast, non-stressed microbial communities are generally considered to have ratios of 0.05 or less (White et al., 1996).

Stress on microbial populations can also result in physiological changes that show an increased concentration of cyclopropyl fatty acids (Guckert et al., 1991). Such changes may be stimulated by starvation, high temperatures, high magnesium ion concentrations and lower pH (Guckert et al., 1986). A cyclopropane PLFA/monoenoic PLFA ratio of greater than 0.1 is indicative of nutritional stress. Cells in the exponential growth phase have cyclopropane PLFA/monoenoic PLFA ratios of less than 0.05 (Smith et al., 2000).

Previous studies have indicated correlation to some extent between PLFA analysis and enzymatic activities (Waldrop, et al., 2000), Biolog®, and DNA analyses (Widmer, et al., 2001). It is however, recommended that PLFA analysis be used in conjunction with and complementary to other techniques (Widmer et al., 2001), such as DGGE.

Notwithstanding the value of this technique, a few important restrictions should be taken into consideration. In a number of cases a specific fatty acid present in a soil sample cannot be linked with a specific microorganism or group of microorganisms, because appropriate signature molecules are not known for all organisms (Hill et al., 2000). Different microbial species can share various fatty acids, therefore PLFA profiles cannot be used to identify species within a community (Hill et al., 2000; Ibekwe et al., 2002). Since the method relies heavily on signature fatty acids to determine gross community structure, any variation in

these signatures would give rise to false community estimates created by artefacts in the methods (Hill et al., 2000).

7. PROBLEM STATEMENT

The South African mining sector provides employment for more than 400 000 people, of which more than 80 000 in the coal mining industry. In 2001 the mining sector accounted for 10% of the countries total gross domestic product (GDP) and 41.5% (R690 billion) of the total market capitalisation of the Johannesburg Securities Exchange. South Africa is the third largest exporter and fourth largest producer of steam coal in the world. In 2001 the coal industries total production amounted to 224 181 171 metric tons and the total sales value to more than R26.5 billion, the third highest of all commodities. Ingwe Mines was responsible for 42 212 941 metric tons of coal sales in 2001, second only to Anglo Operations Ltd (Chamber of Mines of South Africa, 2001).

Even though mining in South Africa contributes to the economy and provides a great deal of employment and training opportunities for local people, the enormous social and environmental impacts caused by mining activities cannot be ignored (Milton, 2001). According to the National State of the Environment Report for South Africa (1999), mining waste constitutes waste rock, tailings (processed material) and polluted process water (DEAT, 1999). Current mining activities generate more than 70 percent of the solid waste produced annually in South Africa (DEAT, 1999). Mine tailings are being processed at a rate of millions of tons per year (Rösner et al., 2001) and discard sites cover large areas of productive land (Van Wyk, 2002). It is thus of great importance to find a sustainable means of mitigating the negative effects associated with mining activities, in the interest of ecosystem health and sustainable land use. Legislation that provides for the restriction of damaging activities to the environment includes the South African Environment Conservation Act (73 of 1989) and the South African Minerals Act (50 of 1991). These laws call for developers to incorporate the cost of ecological rehabilitation into their operational budgets and require rehabilitation to take place as part of and in conjunction with the mining process. Mine closure and reclamation thus need to be planned from the

beginning and executed throughout the mines operation (Hoskin, 2003). According to Hoskin (2003), reclamation is the restoration of land affected by mining to enable, whenever possible, another economic use. In compliance with the mitigation and rehabilitation requirements, many opencast mine rehabilitation projects cover waste rock piles and discard dumps with a layer of topsoil which is excavated from an adjacent borrow pit or stripped from the site before mining (Harris et al., 1989). Agronomic approaches, such as cultivation, fertilisation, reseeding and irrigation have often been adopted for the rehabilitation and revegetation of these sites. This approach has however, failed extensively in the arid areas of southern Africa; primarily due to the lack of the establishment of self-sustainable vegetation cover at these sites, resulting in significant negative environmental consequences (Milton, 2001). The establishment of lasting vegetation cover on mine tailings and discard sites is vital in achieving restoration of these disturbed areas (Carroll et al., 2000). Negative factors that complicate the establishment of vegetation cover include a soil environment typified by poor physical characteristics (Van Wyk, 2002), low levels of plant nutrients and organic matter, pH extremes and the presence of heavy metals (Mining Review Africa, 2003). This is primarily due to the fact that all soil horizons are combined before use as topsoil. In addition, the processing of mine tailings and discard material usually results in an elevated topography which means that these discard sites are particularly exposed to the adverse effects of wind and water erosion (Van Wyk, 2002). These aspects, often accompanied by difficult climatic conditions characteristic to arid and semi-arid areas of southern Africa, deter the establishment of permanent self-sustaining vegetation cover on mine stockpiles and tailings (Milton, 2001; Mining Review Africa, 2003). It is probable that persistent vegetation cover could only be established in conjunction with diverse and self-sustaining biological communities. Clearly, compliance with mitigation and rehabilitation requirements cannot be enforced without a thorough understanding of the ecological principles that ensure ecological stability and subsequent sustainability of ecosystems (Milton, 2001).

Recently, the essence of soil quality in achieving sustainable agronomic, ecological and macro- and microeconomic environments has become apparent, as well as its fundamental role in the establishment of self-sustaining vegetation cover (Masciandaro and Ceccanti,

1999; Marcote et al., 2001). The persistence of vegetation cover on areas under rehabilitative management depends largely on the interaction of revegetated plants with the physical, chemical and biological aspects of the soil profile (Van Wyk, 2002). It is therefore important, when characterising soil quality, to use a selection of all types of soil properties constituting soil quality as a whole. Selected properties should include properties pertaining to chemical, physical and biological aspects of soil and should be those most sensitive to management practices and environmental stress (Hill et al., 2000). Early indicators of changes in soil quality are needed to detect stress and promote long-term sustainability of ecosystems. Chemical and physical parameters change very slowly and therefore many years are required to measure significant changes. On the other hand, soil microbial and biochemical properties are responsive to small changes that occur in the soil, thereby providing immediate and accurate information on the changes in soil quality (Ibekwe et al., 2002).

Soil quality, however, remains difficult to measure because soil and its functions are an ecologically complex phenomenon. It cannot be readily assessed by any single soil parameter, but instead must be evaluated as a function of several independent and/or correlated chemical, physical and biological properties that may exist at different spatial or temporal scales (Doran and Safley, 1997). Industrial and mining companies in South Africa have a social responsibility to ensure that post-land usage capability and subsequently soil quality, should be similar or better than its pre-land use capability as cited in the specific company's environmental management progress report (EMPR). According to Hoskin (2003), the 'objective of mine closure is to leave a mine site in a condition which is safe and stable, and limits further environmental impact so that the mining tenements can be relinquished for alternative land use'. There are certain criteria that have to be adhered to and stipulated as such in the companies Closure Plan. The selection of these criteria must be done in such a manner that a 'balance between costs and benefits of reducing requirements for future care and risk to the environment' is achieved (Robertson and Shaw, 2003). The objective of rehabilitation should be the recreation of sustainable ecosystems based on ecological principles (Van Wyk, 2002). An objective that cannot be attained without thorough consideration of all the complex and dynamic interactions that drive

ecosystems. This places a huge responsibility on companies to minimise social risk and to monitor and improve the quality of the disturbed environment using the best available technology at minimal costs. In this study, a holistic and multidisciplinary approach is proposed that could assist in the successful rehabilitation, and establishment of self-sustaining vegetation covers at industrially disturbed areas, including mine and fly ash disposal sites. Additionally, this approach could negate the negative social and environmental impacts frequently associated with these areas.

8. RESEARCH OBJECTIVES

The aim of this study was to characterise the soil quality of the topsoil covers of seven coal discard sites of different ages and different management histories located within the grassland biome of South Africa. Ingwe Mine Closure Operations are currently managing all of these sites. It is hypothesised that the characterisation of the inter-relationship between the various soil properties and the major environmental factors influencing the biological soil quality should facilitate the selection and implementation of strategic management criteria and the manipulation of operational criteria to enhance the self-sustainability of the vegetation cover and negate the negative social and environmental impacts frequently associated with coal discard sites.

Specific objectives of this study included:

1. Characterisation of the structural diversity of the microbial communities at the various sites by enumeration of specific groups of bacteria and fungi involved in essential aspects of the biogeochemical cycles in soil using selective media and conventional microbiological culturing techniques, as well as analysis and quantification of specific signature lipid biomarkers;
2. Characterisation of the biochemical properties of the topsoil at the various sites by determination of the potential activities of the major enzymes representative of the main steps of soil biogeochemical nutrient cycles, i.e. Carbon (β-glucosidase), Nitrogen

(urease), Phosphorous (acid and alkaline phosphatases) and microbial biomass (dehydrogenase activity);

3. Correlation of the physical and chemical characteristics and percentage ground and crown cover of the vegetation growing at the various sites, with the functional and structural diversity of the microbial communities;
4. Multivariate statistical analysis of the influence of the dominant soil physical and chemical characteristics on the microbial community function and structure;
5. Identification of the predominant physical and chemical parameters that influence the biological and biochemical properties of the soil and subsequent self-sustainability of vegetation cover on these mine-tailing sites; and
6. Recommendation of strategic management criteria for the manipulation of operational criteria based on the soil quality/environmental sustainability indices, to facilitate the establishment of self-sustainable vegetation covers at the coal discard sites.

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CHAPTER 3

SOIL BIOCHEMICAL AND MICROBIOLOGICAL PROPERTIES AS ASSESSMENT CRITERIA FOR THE REHABILITATION OF COAL DISCARD SITES IN SOUTH AFRICA

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ABSTRACT

Soil biochemical properties have been reported to be useful indicators of soil quality and could possibly serve as assessment criteria of successful rehabilitation of ecologically disturbed areas. During this study, the relationship between microbial community function and structure (as characterised by enzymatic activity and selective enumeration, respectively), vegetation cover, and physical and chemical characteristics of the topsoil covers of seven coal discard rehabilitation sites were evaluated. Each sample was characterised by analyses of the physical and chemical characteristics of the topsoil cover; the vegetation cover; dehydrogenase, ß-glucosidase, urease, acid and alkaline phosphatase enzymatic activities and the quantification of aerobic oligotrophic and copiotrophic heterotrophic bacteria and fungi. No significant difference ($p>0.05$) existed between the various sites based on conventional microbial enumeration techniques. The relationship between soil physical and chemical characteristics and the microbiological and vegetation variables was investigated using PCA and RDA multivariate ordination techniques. All enzymatic activities, with the exception of urease and alkaline phosphatase displayed a strong, positive association with the %C. Ammonium concentration had a weak association with all the enzymes studied and pH only showed a negative association with acid phosphatase activity. Based on the results obtained during this study, it is evident that the

absence or low percentage vegetation cover and associated lower organic matter content of the soil have a significant negative impact on soil biochemical properties (enzymatic activity) as well as microbial population size. Results obtained suggest that the quality of the topsoil covering used on the coal discard sites could become more ecologically sustainable for revegetation provided effective management to enhance the organic carbon content. These results indicate that microbial activities can be used as complementary assessment criteria for the evaluation of the success of rehabilitation of mine tailing and discard sites.

Keywords: Microbial activity; Enzymatic activity; Coal discard; Rehabilitation; Soil quality.

1. INTRODUCTION

Soil quality and productivity constitutes a very important natural resource that should not only be preserved, but where possible also improved (Pascual et al., 2000). Under South African legislation, the South African Environment Conservation Act (73/1989) requires developers to ecologically rehabilitate damaged environments, which calls for careful planning and implementation of sound silvicultural and ecological principles. One of the more critical aspects of the rehabilitation process is the improvement of the tailings material to sustain plant growth by the creation of a suitable growth medium.

The South African Minerals Act (50/1991) states that mine closure is among other aspects, dependent on the containment of pollution caused by spoils through soil amelioration and revegetation of mine tailings and discard sites. The coal discard waste produced during coal mining in South Africa is characterised by a high content of pyrite, which under high moisture and oxidation conditions results in the formation of sulphuric acid due to both biological and chemical processes (Bell et al., 2001). This process is largely responsible for the formation of acid mine drainage and poor vegetation growth on mine discard sites. The rate of oxidation in the spoil material will be a function of its particle size distribution, moisture content, and compaction. Neutralisation of sulphuric acid results in the formation of sulphate salts, which culminate into high concentrations of soluble salts. One control measure frequently applied for the containment of discard material is the application of a topsoil cover, which assists in the prevention of further oxidation and facilitates the subsequent revegetation of the site. The establishment of permanent vegetation on most mine waste sites in South Africa is, however, problematic in spite of the annual addition of large quantities of nitrogen and phosphorus fertilisers. This is to a large degree a function of the quality and physical properties of the topsoil cover. Despite the excellent plant cover (both in terms of productivity and frequency) that typifies some rehabilitated areas, uncertainty exists as to the sustainability of the vegetation growth using current management practices, specifically in light of the costs associated with the annual addition of fertilisers.

As soil is a critical component of the biosphere and soil quality is intrinsically linked to overall environmental quality (Marcote et al., 2001), this often leads to specific management strategies and subsequently, amelioration procedures. Soil quality includes a measure of a soil's capacity to function within an ecosystem and to sustain equilibrium through certain processes. Soil functions to produce plant biomass, maintain animal health and production, recycle nutrients, store carbon, partition rainfall, buffer anthropogenic acidity, remediate added animal and human wastes and regulate energy transformations (Doran and Zeiss, 2000). Microbial activity is fundamental in the processes that make energy and nutrients available for recycling in the ecosystem and soil microorganisms play crucial roles in the biogeochemical cycling of carbon (C), nitrogen (N), and phosphorus (P) (Bandick and Dick, 1999; Schoenholtz et al., 2000). Poor management practices and other negative impacts on soil ecosystems result in the loss of functional groups of microorganisms and subsequent ecosystem modification or even collapse (Hawksworth, 1996). Changes due to management practices generally affect the functional and structural properties of soil and can be measured either directly or indirectly (Masciandaro and Ceccanti, 1999). Consequently, the monitoring of rehabilitation success and therefore indirectly soil quality and fertility relies on the use of indicators.

An indicator in this case would be a measurable soil attribute that determines how well a soil functions (Schoenholtz et al., 2000), or if a specific management strategy has a positive or negative influence on soil quality. Traditionally, physical and chemical analyses of soil such as pH, water holding capacity and total carbon and nitrogen contents have often been used in the characterisation of soil and therefore formed the basis for most management decisions. Chemical and physical analyses are, however, slow to change and the response of these parameters can only be measured effectively over an extensive period (Pascual et al., 2000). Furthermore, soil is a very complex ecosystem and the analysis of physical and chemical characteristics does not allow conclusions to be made concerning the biological structures and functions within the soils (White et al., 1996; Hill et al., 2000). Microbiological and biochemical properties of soil have often been proposed as early and sensitive indicators of soil ecological stress or restoration processes because they are responsive to small changes that occur in soil (Pascual et al., 2000; Badiane et al., 2001;

Ibekwe et al., 2002). Previously, the analysis of soil microbial communities has relied extensively on culture dependent techniques using a variety of enriched culture media. These conventional microbiological techniques are, however, insensitive and provide little insight into the nutritional and/or environmental status *in situ* (White et al., 1996).

Enzyme assays are process level indicators and a culture-independent method with results presented as a means of determining the potential of a soil to degrade or transform substrates (Dick et al., 1996). The presence and activity of enzymes is vital for all biochemical transformations in soil, thus the study of soil enzymatic activities provides insight into microbial dynamics and populations (Riffaldi et al., 2002). The measurement of a variety of enzymes in soil gives an indication of the diversity of functions that can be assumed by the microbial community (Brohon et al., 2001). According to Dick et al. (1996), the conceptual rationale for using soil enzyme activity as a soil quality indicator is that enzyme activities: (i) are often closely related to important soil quality parameters such as organic matter, soil physical properties and microbial activity or biomass; (ii) can begin to change much sooner (1 to 2 years) than other properties (e.g., soil organic carbon) thus providing an early indication of the trajectory of soil quality with changes in soil management; (iii) can be an integrative soil biological index of past soil management; and (iv) involve procedures that are relatively simple compared to other important soil quality properties (e.g., physical, chemical and some biological measurements). Soil enzymes have therefore been suggested as potential indicators of soil quality because of their essential role in soil biology, ease of measurement and rapid response to changes in soil management (Bandick and Dick, 1999; Riffaldi et al., 2002). Soil enzymes representative of the main biogeochemical cycles (C, N, P, S) and of microbial biomass are often used as indicators of soil health. Enzymes often studied in this context are β-glucosidase (carbon cycle), urease (nitrogen cycle), and phosphatase (phosphorus cycle) (Pascual et al., 2000; Trasar-Cepeda et al., 2000; Aon et al., 2001a,b). Studies have shown that enzymatic activities such as that of dehydrogenase, β-glucosidase, urease, and phosphatase show significant correlation with total organic carbon (TOC), total nitrogen (TN), water-filled pore space (WFPS), and heterotrophic bacterial and fungal biomass (Bandick and Dick, 1999; Aon and Colaneri, 2001).

In this paper the relationship between physical and chemical characteristics of topsoil used during the rehabilitation and soil biochemical properties, specifically the functional and structural diversity of the microbial communities present within the topsoil cover, was assessed in order to establish the soil quality and subsequent sustainability of the revegetation of the topsoil covers of various coal discard sites under rehabilitation.

2. MATERIALS AND METHODS

2.1. Site details

The study was conducted on seven already vegetated coal discard sites that are under rehabilitation and managed by Ingwe Closure Operations, Ingwe Mines, South Africa (site identities are presented in Table 1). The topsoil used as cover was excavated from adjacent borrow pits or stripped from the sites before mining. All the coal discard sites were vegetated with a grass seed mixture mostly dominated by the commercially available grasses *Eragrostis tef, Eragrostis curvula, Chloris gayana, Digitaria eriantha, Cynodon dactylon* and *Pennisetum clandestinum.* As a management practice, all dumps are regularly defoliated through mowing and/or cattle grazing, the latter being the preferred practice. All seven discard sites were also treated with variable amounts of lime, inorganic fertilisers, and well-cured kraal manure at the onset of rehabilitation and during annual maintenance. As there were no suitable control sites, random sites from the surrounding areas, representative of relatively natural veldt, were chosen as reference sites. These sites were located in areas adjacent to three of the coal discard sites examined (Table 1). All sites were situated within the summer rainfall area of South Africa and receive an average annual rainfall between 700-800 mm. All the sites were located within the Grassland biome.

2.2. Sampling procedure

A random sampling design was used to obtain three composite samples per site ($n = 21$) of the topsoil from the seven coal discard sites (Alef and Nannipieri, 1995; Dick et al., 1996).

Composite samples were also obtained from three sites (n = 15) in presumed undisturbed areas to represent natural grassland ecosystems (reference sites). All samples were obtained during November 2002 in the same quadrates used for the assessment of vegetation growth. A soil auger was used to obtain volume samples and a minimum of 1 kg of soil per sampling area was obtained. Sampling was only conducted in the top 0-15 cm of the topsoil layer, since it has previously been reported that the number of bacteria and microbial activity decline with depth (Aon and Colaneri, 2001; Taylor et al., 2002). In addition, most of the dumps had an effective topsoil cover depth of only 10-15 cm and because of the compaction within the coal discard to prevent spontaneous combustion as well as unfavourable growth conditions, most of the plant root biomass should only be located within the 10-15 cm topsoil layer. Aseptic techniques were used as described by Dick et al. (1996). Soil samples were placed in tightly sealed plastic bags and kept at 4°C to keep them field moist and to preserve biological properties. Composite (consolidated) samples were mixed thoroughly to contain equal amounts (weight) of individual samples from the same depth (Alef and Nannipieri, 1995). Each consolidated sample was analysed separately. The chemical and physical analyses for the characterisation of topsoil cover currently on the respective sites were done as previously described (Van Rensburg et al., 1998). Twenty-four soil chemical and physical variables were analysed during this study.

2.3. Vegetation coverage

The ground and crown vegetation coverage of the rehabilitation and reference sites respectively, was visually estimated in three 1 m^2 quadrates randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all grass species over a fixed area. Both values are expressed as a percentage per surface area (1 m^2).

2.4. Determination of soil dry mass

The weight of each soil sample was determined in its field moist state and again after oven drying (105°C overnight). The difference attributed to the loss of water was calculated and related to the soil moist weight. The dry mass and soil moisture content (% soil moisture) was determined as reported by Alef and Nannipieri (1995).

2.5. Microbial counts

Counts of colony forming units (CFUs) of bacteria and fungi in the topsoil were determined by plating the dilution series onto selective media. Total heterotrophic bacteria were enumerated using cycloheximide agar, R2A, and soil extract agar (SEA). Sodium pyrophosphate ($Na_2PO_4 \cdot 7H_2O$; pH 7.0) (0.1% m/v) solution was used as diluent during the enumeration of bacteria (Frederickson and Balkwill, 1998). Soil extract agar and R2A function as selective media for oligotrophic and copiotrophic heterotrophic bacteria, respectively (Taylor et al., 2002). A separate SEA was prepared for each of the seven topsoil cover samples investigated during this study. According to the procedure as described by Fredrickson and Balkwill (1998), fungi were enumerated by plating the dilution series onto rose bengal-streptomycin agar (RBS Agar). Dextrin (0.2% m/v) and dextrose (0.2% m/v) solutions were used as diluents for suspension of the topsoil and dilution, respectively (Alef and Nannipieri, 1995). For the enumeration of bacteria and fungi, 0.1 ml of the 10^{-1} to 10^{-8} dilutions of field-moist soil were plated onto the respective media. All plates were incubated at room temperature (22±3°C) for ca. 4-10 days. All microbial enumerations were carried out in duplicate.

2.6. Measurement of soil enzymatic activities

Before analyses, consolidated soil samples were passed through a 2 mm sieve. For the determination of dehydrogenase activity, soil was kept field moist, while air-dried samples were used for determination of β-glucosidase (EC 3.2.1.21), urease (urea amidohydrolase, EC 3.5.1.5), and acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5)

and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatases activities (Alef and Nannipieri, 1995). All analyses were carried out in triplicate.

2.6.1. Dehydrogenase activity

Dehydrogenase activity is indicative of microbial activity (Nannipieri et al., 1996) and was estimated according to the procedure as described by Alef and Nannipieri (1995). Field moist soil (1.0 g) was weighed into 50 ml screw-cap Erlenmeyer flasks and incubated in the dark for 2 h at 40°C with 1.5 ml Tris (hydroxy methyl)-aminomethane buffer and 2 ml iodonitrotetrazolium chloride (INT) (5 mg ml^{-1} in 2% v/v *N,N*-dimethylformamide). Controls were performed with sterilised (1.0 g samples, autoclaved at 121°C for 20 min) soil. The reaction was terminated by the addition of 10 ml *N,N*-dimethylformamide/ethanol (1:1 v/v) extractant and shaking at 20 min intervals for 1 h. The soil suspension was filtered through Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 464 nm. The dehydrogenase activity was expressed as μg INF g^{-1} dry weight $2h^{-1}$.

2.6.2. β-glucosidase and phosphomonoesterase activity

β-glucosidase (EC 3.2.1.21) and acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase activities were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively) (Dick et al., 1996). For the β-glucosidase assay, 1.0 g soil (air dried) was placed in 50 ml screw-cap Erlenmeyer flasks and incubated for 1 h at 37°C with 0.25 ml toluene, 4 ml modified universal buffer (pH 6.0) and 1 ml *p*-nitrophenyl-β-D-glucosidase (PNG). The reaction was terminated by the addition of 1 ml 0.5 M calcium chloride ($CaCl_2$) and 4 ml 0.1 M Tris (hydroxy methyl)-aminomethane buffer (pH 12.0). Controls were performed by adding substrate immediately after incubation, before the addition of $CaCl_2$ and Tris-buffer. The soil suspension was immediately filtered through Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 410 nm. β-glucosidase activity was expressed as

mg *p*-nitrophenol g^{-1} dry weight h^{-1}. Acid (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, pH 6.5) and alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) phosphatase activities were assayed using the method described by Alef and Nannipieri (1995).

Phosphomonoesterase assays differed from the above only in the choice of buffer. Modified universal buffer pH 6.5 and pH 11.0 were used for acid and alkaline phosphomonoesterase, respectively. Phosphatase activity was expressed as mg *p*-nitrophenol g^{-1} dry weight h^{-1}.

2.6.3. Urease activity

Urease (urea amidohydrolase, EC 3.5.1.5) activity was assayed using the procedure as described by Alef and Nannipieri (1995). Air-dried soil (5.0 g) was incubated with 2.5 ml urea solution at 37°C for 2 h. After incubation, 50 ml of 1.0 M potassium chloride (KCl) solution was added and the flasks shaken for 30 min. The soil suspensions were filtered through Whatman no. 2 filter paper and the absorbance of the filtrate measured at 600 nm. Controls were prepared with 2.5 ml distilled water and the urea solution was added at the end of the incubation, immediately before the addition of the KCl solution. Urease activity was expressed as μg NH_4-N g^{-1} dry weight 2 h^{-1}.

2.7. Statistical analysis

Parametric and non-parametric statistical analyses were performed on all data obtained using STATISTICA 6 (StatSoft, Inc ©). The data was tested for normality using the Shapiro-Wilk's test. In the case of data being normally distributed (parametric) a breakdown and one-way ANOVA was performed and the Tukey's honest significant difference (HSD) test was used to determine statistical significance between the various samples. In the case of non-parametric data, non-parametric data analysis was performed and the Kruskal-Wallis ANOVA and Median test was used to determine statistically significant difference between samples. In all cases, there were no differences between the

results obtained from the respective normality analyses. As a result, only the parametric analyses of the relevant statistics are discussed.

The relationship between soil physical and chemical characteristics, the microbiological and the vegetation variables was investigated using Principal Components Analysis (PCA) and Redundancy Analysis (RDA) multivariate ordination techniques using CANOCO (Canoco for Windows Version 4.0, GLW-CPRO ©). Principal Components Analyses were conducted on the soil physical and chemical variables, as well as on the microbial enzymatic activities, microbial counts obtained using conventional techniques and the percentage ground and vegetation crown coverage in order to determine how these variables were inter-correlated thereby to assess for multicollinearity between the variables. An Redundancy Analysis (RDA) was subsequently performed with the activities of the five enzymes assayed, the microbial counts obtained using conventional techniques and the percentage ground and crown coverage as species dependent variables and the most significant soil variables as independent environmental factors. The most significant soil physical and chemical variables were selected through the forward selection procedure provided in CANOCO, thereby ensuring that only the most pertinent environmental gradients were investigated.

3. RESULTS AND DISCUSSION

The status of nine microbiological assays (five enzymatic and copiotrophic and oligotrophic heterotrophic bacterial and fungal counts on four different media), percentage ground and crown vegetation coverage, as well as 24 different physical and chemical parameters of seven distinct vegetated coal discard sites and three reference sites were investigated during this study.

3.1. Sites under rehabilitation

3.1.1. Physical and chemical characteristics of the various topsoil covers

Results obtained for the physical and chemical characterisation of the topsoil samples from the various coal discard sites are summarised in Table 1. A PCA ordination diagram illustrating the relationship between the physical and chemical characteristics of the topsoil cover layer at the various rehabilitation sites is presented in Figure 1. Based on these results, it can be concluded that the topsoil used as cover at the various coal discard sites varied markedly between sites based on physical and chemical characteristics.

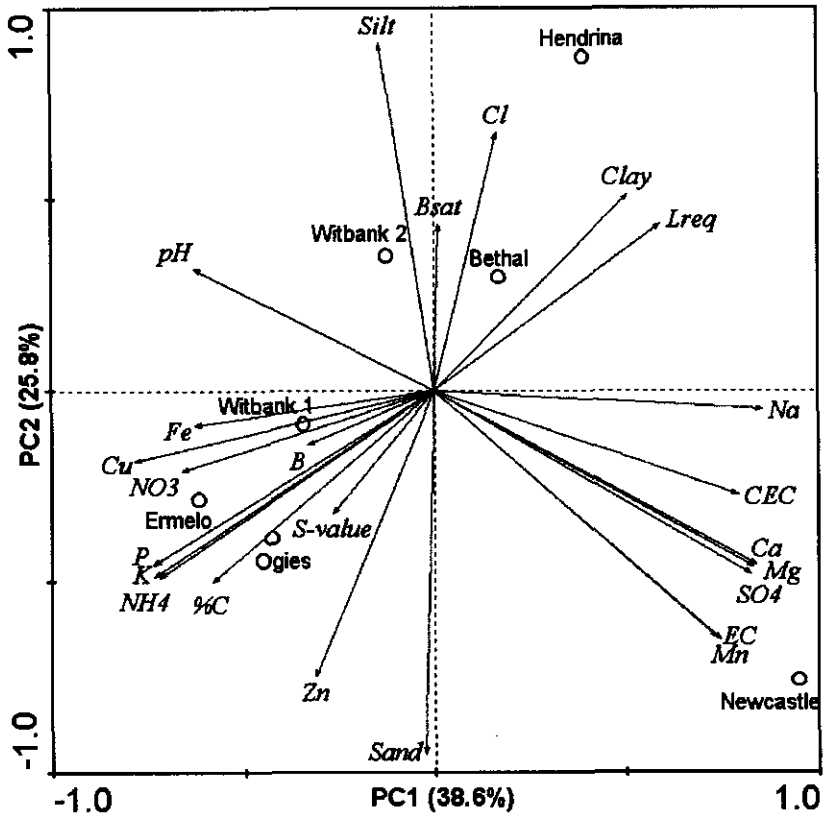

Figure 1. Principal Components Analysis (PCA) ordination diagram of the physical and chemical characteristics of the topsoils used as cover at the various coal discard sites. The eigenvalues for the first two ordination axes of the PCA were 0.386 and 0.258, respectively. These two axes accounted for 64.5% of the total observed variance.

Table 1. Physical and chemical properties of the topsoil covers obtained from the seven coal discard sites and three reference sites.

Properties	Rehabilitation sites							Reference sites		
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies	Newcastle	Ermelo	Ogies
Grid Reference	27°51'14"S 29°57'23"E	26°29'41"S 29°45'34"E	26°20'4"S 29°53'19"E	26°10'8"S 29°21'2"E	25°53'42"S 29°10'27"E	25°54'35"S 29°11'21"E	26°01'22"S 29°06'06"E	27°50'S 29°56'E	26°19'S 29°35'E	26°00'S 29°04'E
Rehabilitation age (years)	8	3	4	1	4	4	4	N/A	N/A	N/A
Ca ($mg\ kg^{-1}$)	49.30 ± 13.04	7.36 ± 0.27	3.73 ± 1.68	25.10 ± 9.72	2.92 ± 0.53	3.13 ± 0.27	5.65 ± 1.29	0.05 ± 0.02	0.22 ± 0.17	0.10 ± 0.02
Mg ($mg\ kg^{-1}$)	15.77 ± 3.40	2.08 ± 0.22	1.47 ± 0.64	7.89 ± 4.41	0.92 ± 0.18	0.98 ± 0.16	1.22 ± 0.49	0.19 ± 0.02	0.33 ± 0.15	0.15 ± 0.01
K ($mg\ kg^{-1}$)	6.29 ± 1.71	5.31 ± 0.78	25.08 ± 8.32	5.90 ± 1.96	4.92 ± 1.26	12.59 ± 6.51	24.59 ± 9.31	0.21 ± 0.01	0.18 ± 0.07	0.55 ± 0.14
Na ($mg\ kg^{-1}$)	2.08 ± 0.30	1.85 ± 0.12	1.21 ± 0.44	1.56 ± 0.44	0.93 ± 0.12	0.87 ± 0.27	1.21 ± 0.17	0.13 ± 0.07	0.05 ± 0.01	0.03 ± 0.00
SO_4 ($mg\ kg^{-1}$)	239.00 ± 72.57	28.27 ± 2.17	13.77 ± 4.72	122.04 ± 71.69	25.62 ± 12.21	26.34 ± 6.06	32.62 ± 15.90	0.16 ± 0.03	0.18 ± 0.06	0.19 ± 0.04
NO_3 ($mg\ kg^{-1}$)	2.45 ± 1.23	6.38 ± 1.30	16.19 ± 6.59	6.38 ± 5.15	1.31 ± 0.16	6.05 ± 3.50	11.45 ± 7.31	0.08 ± 0.04	0.24 ± 0.18	0.25 ± 0.03
NH_4 ($mg\ kg^{-1}$)	0.39 ± 0.07	0.26 ± 0.07	0.73 ± 0.23	0.30 ± 0.04	0.43 ± 0.09	0.64 ± 0.07	0.47 ± 0.21	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.00
Cl ($mg\ kg^{-1}$)	2.59 ± 0.18	12.75 ± 5.20	5.71 ± 1.96	5.35 ± 0.71	1.69 ± 0.32	4.01 ± 1.35	3.03 ± 1.03	0.30 ± 0.09	0.18 ± 0.08	0.33 ± 0.11
Fe ($mg\ kg^{-1}$)	33.44 ± 18.89	81.48 ± 16.06	5659.82 ± 2273.28	105.93 ± 89.03	5937.56 ± 2048.50	11956.04 ± 743.35	2126.28 ± 878.29	23.12 ± 1.64	21.52 ± 3.61	43.05 ± 13.26
Mn ($mg\ kg^{-1}$)	837.25 ± 480.20	16.45 ± 15.02	47.96 ± 14.59	5.25 ± 5.05	19.90 ± 5.57	75.32 ± 6.21	29.99 ± 6.24	2.56 ± 0.42	2.20 ± 0.90	3.49 ± 0.63
Cu ($mg\ kg^{-1}$)	0.96 ± 0.96	3.99 ± 3.99	31.29 ± 4.05	6.86 ± 1.94	8.14 ± 8.14	19.63 ± 8.16	8.62 ± 5.44	0.37 ± 0.22	0.88 ± 0.42	1.25 ± 0.30
Zn ($mg\ kg^{-1}$)	16.94 ± 9.69	6.74 ± 6.50	19.08 ± 4.42	5.59 ± 2.77	4.28 ± 3.79	21.88 ± 6.96	17.27 ± 0.57	0.95 ± 0.14	1.36 ± 0.76	1.31 ± 0.12
B ($mg\ kg^{-1}$)	35.36 ± 35.36	2.72 ± 2.72	0.00 ± 0.00	32.64 ± 16.32	130.54 ± 64.76	89.75 ± 63.38	127.83 ± 67.34	30.83 ± 7.24	22.50 ± 10.52	35.79 ± 2.28
P ($mg\ kg^{-1}$)	18.97 ± 7.53	5.60 ± 0.84	146.43 ± 65.07	18.23 ± 9.75	27.37 ± 7.27	38.13 ± 16.77	96.77 ± 17.27	7.01 ± 1.70	5.51 ± 0.69	17.98 ± 0.25
pH	5.51 ± 0.27	6.20 ± 0.26	7.03 ± 0.030	7.41 ± 0.44	7.15 ± 0.50	6.24 ± 0.41	6.90 ± 0.37	5.74 ± 0.30	6.66 ± 0.48	5.60 ± 0.05
Electrical Conductivity (EC) ($mS\ m^{-1}$)	117.33 ± 46.25	21.67 ± 4.10	29.00 ± 6.27	37.00 ± 4.36	26.33 ± 2.03	18.67 ± 6.23	35.00 ± 8.02	23.00 ± 3.27	29.75 ± 10.90	24.00 ± 3.61
Sand (%)	79.22 ± 5.53	39.80 ± 2.62	68.18 ± 2.53	58.70 ± 1.00	61.23 ± 1.62	68.12 ± 2.61	67.46 ± 0.91	55.57 ± 4.17	58.09 ± 11.12	80.73 ± 3.68
Silt (%)	8.46 ± 1.96	42.66 ± 1.76	23.42 ± 2.84	34.27 ± 1.54	26.27 ± 2.61	20.93 ± 2.92	25.25 ± 2.83	23.67 ± 4.48	14.04 ± 4.09	9.43 ± 2.27
Clay (%)	12.31 ± 3.81	17.55 ± 0.86	8.39 ± 0.51	7.02 ± 1.63	12.51 ± 1.10	10.95 ± 1.75	7.29 ± 2.26	20.76 ± 1.29	27.87 ± 7.48	9.84 ± 1.54
% Organic Carbon (%C)	0.69 ± 0.14	17.55 ± 1.91	2.20 ± 0.74	0.31 ± 0.16	0.32 ± 0.08	0.67 ± 0.01	2.72 ± 1.36	2.98 ± 0.77	2.44 ± 0.75	1.48 ± 0.43
Cation Exchange Capacity (CEC) $cmol(+).kg^{-1}$	24.34 ± 1.60	17.55 ± 1.92	9.34 ± 1.56	7.06 ± 1.77	4.19 ± 0.61	4.90 ± 1.01	9.92 ± 3.95	13.97 ± 3.29	22.35 ± 8.39	6.36 ± 0.95
S-value $cmol(+).kg^{-1}$	13.12 ± 0.17	12.80 ± 3.59	9.58 ± 2.81	8.46 ± 3.43	4.18 ± 1.07	3.23 ± 1.20	95.17 ± 14.20	8.76 ± 2.68	19.74 ± 8.06	3.50 ± 0.91
Base Saturation (Bsat) (%)	54.33 ± 3.07	70.13 ± 12.70	98.94 ± 15.35	112.29 ± 17.54	96.49 ± 13.94	61.28 ± 13.34	6.90 ± 0.37	56.43 ± 6.78	81.66 ± 11.51	53.34 ± 5.67
Lime requirement (Lreq) ($t.ha^{-1}$)	3.55 ± 2.21	8.05 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.62 ± 0.62	1.62 ± 1.04	0.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
% Soil Moisture	6.61 ± 0.35^{ac}	9.87 ± 2.13^{c}	6.84 ± 0.57^{ac}	4.35 ± 0.27^{ab}	1.68 ± 0.04^{b}	2.59 ± 0.29^{ab}	3.02 ± 0.75^{ab}	10.50 ± 1.45^{a}	9.26 ± 2.11^{a}	3.79 ± 1.31^{a}
% Ground Cover	77.42 ± 17.71^{abc}	63.21 ± 23.72^{a}	71.25 ± 19.10^{cd}	41.57 ± 15.67^{ab}	28.92 ± 12.30^{ab}	86.75 ± 16.59^{d}	60.91 ± 14.97^{bcd}	59.00 ± 11.00^{x}	59.00 ± 12.59^{x}	100.00 ± 0.00^{x}
% Crown Cover	56.29 ± 19.85^{b}	38.75 ± 12.36^{b}	80.13 ± 12.95^{b}	45.71 ± 15.97^{a}	42.50 ± 22.11^{a}	84.83 ± 14.27^{b}	65.91 ± 19.98^{ab}	67.00 ± 8.89^{xy}	52.50 ± 9.68^{x}	100.00 ± 0.00^{y}

[1] Values given are mean ± standard error

[2] Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

3.1.2. Vegetation coverage

The percentage ground and crown vegetation cover as was evident during the time of sampling of the topsoil from the various coal discard sites are summarised in Table 1. The highest percentage ground and crown cover was observed at the Hendrina and Witbank 2 sites. This could possibly be attributed to the elevated levels of phosphorus and ammonium in the topsoil covers of these sites (Table 1).

The percentage ground and crown vegetation cover at the Bethal and Witbank 1 sites were significantly lower ($p<0.05$) than that of the other sites, primarily due to the dominance of large tufted grasses with a large crown cover and a smaller ground cover at this site. These sites were also characterised by very low percentages of organic material as well as a low percentage of stoloniferous grasses. The percentage crown cover at the Ermelo site was also significantly lower ($p<0.05$) than the other sites. This can be ascribed to excessive grazing of this site by cattle. A large percentage of organic matter was also present on the soil surface at the Ermelo site due to wastage during grazing activities. Reduced aboveground plant diversity as a result of tillage, overgrazing, pollutants and pesticides has been reported to decrease the microbial diversity in the soil ecosystem and to disturb its normal functioning (Aon et al., 2001a; Garcia et al., 2002). A degraded plant cover generally results in lower soil organic matter (SOM) content. This would imply a lower microbial activity due to the decreased availability of organic matter for decomposition (Garcia et al., 2002).

3.1.3. Microbial counts

The CFUs obtained for the different media are summarised in Table 2. Statistical analysis of the microbial counts indicated no significant difference ($p>0.05$) between the various sites based on conventional microbial enumeration (Table 2).

Table 2. Microbial counts and enzymatic properties of topsoil covers obtained from the seven coal discard sites and three reference areas.

Properties	Rehabilitation sites							Reference sites		
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies	Newcastle	Ermelo	Ogies
Microbial Counts (log CFU g^{-1} dry soil)										
Soil extract agar*	6.29 ± 6.05^{a}	5.23 ± 4.84^{a}	6.17 ± 6.08^{a}	5.17 ± 4.82^{a}	6.35 ± 6.09^{a}	6.18 ± 5.89^{a}	6.86 ± 6.75^{a}	6.26 ± 0.08^{x}	6.92 ± 0.24^{x}	6.07 ± 0.10^{x}
R2A*	7.03 ± 6.69^{a}	7.26 ± 7.05^{a}	7.61 ± 7.39^{a}	7.70 ± 7.34^{a}	6.67 ± 6.60^{a}	7.33 ± 7.30^{a}	6.56 ± 6.26^{a}	5.09 ± 0.94^{y}	4.96 ± 0.16^{xy}	3.73 ± 0.51^{x}
Cycloheximide*	6.66 ± 6.47^{a}	6.88 ± 6.45^{a}	7.70 ± 7.49^{a}	7.30 ± 7.16^{a}	8.37 ± 8.37^{a}	7.09 ± 6.72^{a}	6.63 ± 6.57^{a}	6.82 ± 0.17^{x}	6.74 ± 0.12^{x}	6.47 ± 0.04^{x}
Rose Bengal-Streptomycin agar*	4.49 ± 4.15^{a}	3.82 ± 3.47^{a}	4.54 ± 3.34^{a}	4.04 ± 3.27^{a}	3.13 ± 2.38^{a}	3.28 ± 2.66^{a}	3.82 ± 3.47^{a}	7.31 ± 0.08^{z}	7.12 ± 0.19^{yz}	6.76 ± 0.10^{xy}
Enzymatic activities										
Dehydrogenase* (µg INF g^{-1} $2h^{-1}$)	285.92 ± 159.45^{a}	315.83 ± 42.63^{ad}	578.51 ± 222.38^{b}	139.93 ± 22.68^{a}	67.53 ± 28.69^{a}	107.80 ± 40.38^{ac}	316.00 ± 237.53^{ad}	482.38 ± 143.22^{x}	337.50 ± 60.27^{x}	302.62 ± 62.95^{x}
ß-Glucosidase** (mg PNP g^{-1} h^{-1})	129.76 ± 64.85^{a}	99.51 ± 32.16^{ab}	143.20 ± 41.83^{a}	59.72 ± 25.36^{b}	62.08 ± 47.47^{b}	88.70 ± 12.66^{a}	138.77 ± 63.62^{a}	356.21 ± 79.41^{x}	433.03 ± 153.60^{x}	262.386 ± 84.21^{x}
Alkaline Phosphatase** (mg PNP g^{-1} h^{-1})	352.11 ± 33.09^{a}	453.55 ± 68.87^{ab}	264.64 ± 55.24^{ac}	$391.20 \pm 28..11^{ab}$	221.37 ± 48.07^{ac}	223.29 ± 30.25^{ac}	428.73 ± 313.57^{ab}	718.684 ± 248.11^{x}	675.312 ± 146.89^{x}	564.435 ± 323.36^{x}
Acid Phosphatase** (mg PNP g^{-1} h^{-1})	430.06 ± 149.319^{a}	343.46 ± 43.71^{a}	569.53 ± 80.40^{ab}	343.38 ± 54.49^{a}	404.60 ± 117.25^{a}	344.14 ± 53.33^{a}	687.22 ± 182.05^{a}	1294.30 ± 680.54^{x}	1239.76 ± 95.82^{x}	1358.86 ± 30.54^{x}
Urease** (µg NH_4-N g^{-1} $2h^{-1}$)	49.20 ± 1.37^{a}	58.95 ± 25.02^{ab}	58.48 ± 8.64^{ab}	34.59 ± 5.03^{ac}	41.90 ± 11.22^{a}	34.93 ± 3.92^{ac}	66.94 ± 15.26^{ab}	30.62 ± 5.50^{x}	20.36 ± 7.33^{x}	16.25 ± 1.09^{x}

[1] All values ± SEM represents the results obtained from three independent samples ($n = 3$) at a sampling depth of 0-15 cm.

[2] *Significant at the 0.05 probability level

[3] **Significant at the 0.001 probability level

[4] Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

[5] INF: iodonitrotetrazolium chloride-formazan; PNP: para-nitrophenol.

3.1.4. Enzymatic activities

Enzymatic activity in the soil environment is a major contributing factor to overall soil microbial activity (Garcia et al., 2002). Dehydrogenase, β-glucosidase, alkaline phosphatase, acid phosphatase, and urease activity within the topsoil covers of the seven coal discard sites were assayed during this study because of their vital role in soil microbial activity and substrate mineralisation. These enzymes are responsible for specific hydrolytic reactions and act as catalysts for reactions involved in the biogeochemical transformations of C, N and P, respectively (Aon and Colaneri, 2001; Taylor et al., 2002).

Dehydrogenase activity was assayed as an estimation of overall microbial activity and it has been reported to generally be directly related to organic carbon content of soil (Bandick and Dick, 1999; Aon and Colaneri, 2001). Dehydrogenase activity is considered as an accurate measure of overall microbial activity because it is present in all microorganisms and it has been shown that the type of substrate can influence the dehydrogenase activity, with lower values being observed in acidic soils (Taylor et al., 2002). β-Glucosidase activity is related to the carbon cycle and is very useful in monitoring of soil quality due to several reasons. It fulfils a central role in the cycling of organic matter, is the most abundant of the three enzymes involved in cellulose degradation and is rarely substrate limited (Turner et al., 2002). Urease and phosphatase are often measured because of their importance in the nitrogen and phosphorus cycles, respectively (Aon and Colaneri, 2001). Enzymatic activities in relation to the cycling of nitrogen (ammonification, nitrification, denitrification) or phosphorus (release of inorganic phosphorus) in soil have been used to evaluate the fertility of the soil or to describe the functioning of the ecosystem (Aon and Colaneri, 2001; Brohon et al., 2001).

The average activities of the enzymes assayed are presented in Table 2. According to the one-way ANOVA, the enzymatic activities assayed during this study showed an overall statistically significant difference among the sites under investigation at a probability level of 0.05 or less (Table 2). These results suggest that a slight to moderate variation in pH is not a critical factor governing the predominance of acid or alkaline phosphatase activity in

these ecosystems. These results are in contradiction to the reports of Dick and Tabatabai (1984) who stated that acid phosphatase activity predominated in acidic soils, while alkaline phosphatase activity predominated in neutral or alkaline soils. Results for the phosphomonoesterase activity, however, do not discriminate between sites in the same manner as dehydrogenase activity. The reason for this could be that phosphomonoesterases are both intracellular and extracellular enzymes; the extracellular fraction being less sensitive to environmental conditions that affect the physiological state of the microorganisms (Nannipieri et al., 1996).

An RDA ordination diagram illustrating the association between the dominant environmental variables, microbial enzymatic activities, microbial counts and percentage ground and crown cover is presented in Figure 2. The aim of this analysis was to directly relate the most important environmental factors to "species data". The soil variables pH, ammonium (NH_4), nitrate (NO_3), phosphorus (P), and percentage organic carbon (%C) (Table 1) were used in the RDA analysis since these environmental variables explained the enzyme activities the best during the forward selection procedure with the Monte Carlo Permutation Test using CANOCO.

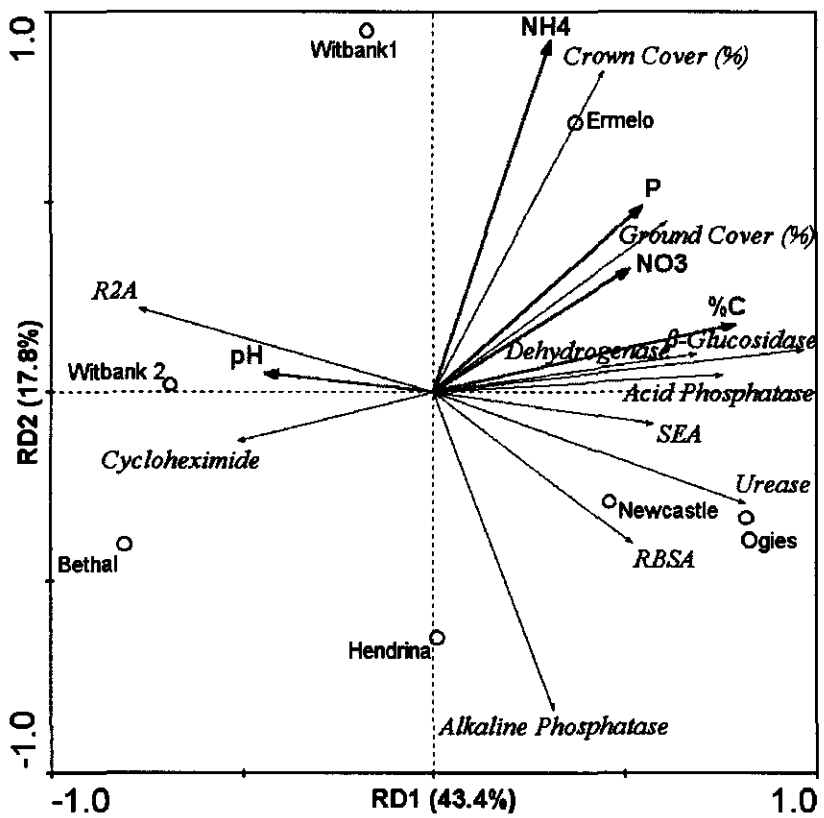

Figure 2. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables, soil microbiological enzymatic activities, bacterial and fungal colony forming units quantified on different media as well as the ground and crown vegetation coverage of the topsoil covers of the coal discard sites. Enzymatic activity, bacterial and fungal colony forming units and percentage ground and crown vegetation coverage are represented by solid vectors and the environmental physical/chemical parameters by dashed vectors. Eigenvalues for the first three axes were 0.434, 0.178 and 0.145, respectively. Total observed variance of the first two canonical axes was 65.3%. The first canonical axis correlated strongly with percentage organic carbon (%C) (r^2 = -0.8023), the second axis correlated with NH_4^+ (r^2 = 0.9230) and the third axis (not shown) correlated with pH (r^2 = -0.8700). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis as well as the overall effect of the chosen environmental variables on the microbial enzymatic activities was statistically significant (p = 0.02 and p = 0.005, respectively).

Based on the results obtained, it is evident that the dominant chemical characteristics of the topsoil used as cover at the various coal discard sites varied significantly between sites. Furthermore, dehydrogenase, β-glucosidase, acid phosphatase and urease activities were

strongly associated with the percentage carbon in the topsoil covers. These enzymes were also positively associated with the fluoride extractable phosphorus (Bray 1) and nitrate content of the topsoil covers, but to a lesser extent. Ammonium was moderately associated with dehydrogenase, β-glucosidase, and acid phosphatase activities. However, no association was observed between urease activity and the ammonium concentration in the soil. These results confirm the observations of Garcia et al. (2002).

Alkaline phosphatase activity was negatively associated with ammonium, nitrate and phosphorus concentrations in the topsoil covers. A negative association was also apparent between pH and all the enzymatic activities assayed during this study. Soil pH impacts on plant response by altering the equilibrium solid phase, the availability of nutrients, the composition and diversity of the microbial communities present in soil and subsequent enzymatic activity (Dick et al., 2000).

During this study, dehydrogenase activity was always high and positively associated with oligotrophic bacterial and fungal counts, as was also the case with alkaline phosphatase activity. Urease was also observed to associate negatively with copiotrophic bacterial counts and positively with fungal counts, confirming the results reported by Aon et al. (2001a). A stronger association was apparent between oligotrophic bacterial counts than fungal counts and enzymatic activity.

Rehabilitation sites to the left of the first ordination axis (Newcastle, Ermelo, Hendrina and Ogies) (Figure 2) had higher soil microbiological and biochemical activities as well as percentage ground and crown vegetation cover than the sites (Bethal, Witbank 1 and Witbank 2) to the right of this axis. This is probably related to the higher organic carbon content of these sites. The highest soil microbiological and biochemical activities were observed in the topsoil cover obtained from the Ogies coal discard site. The low dehydrogenase, β-glucosidase, acid phosphatase and urease activities observed at the Bethal, Witbank 1 and Witbank 2 sites indicate lower overall microbial activity at these sites. These sites also had the lowest organic carbon content and percentage ground and crown vegetation coverage. Consequently, it can be assumed that the soil quality and

fertility of the topsoil covers at these sites are lower because of decreased microbial activity due to the deficit of biodegradable organic matter and limited nutrient cycling. The opposite applies to sites with higher dehydrogenase β-glucosidase, acid phosphatase and urease activities. The fact that both dehydrogenase and β -glucosidase activity positively corresponded with organic carbon content of the soil (Figure 2) emphasises the importance of organic matter as soil amendment during rehabilitation. These results are in accordance with results obtained by Garcia et al. (2002) and indicate that the rehabilitated areas at Hendrina and Ogies possibly had the highest turnover of nutrients in the ecosystems. Garcia et al. (2002) also reported that the lowest phosphatase activities were observed in the soil with less plant cover. During this study, a negative association between acid phosphatase activity and plant cover was also observed. Alkaline phosphatase activity, however, showed a slight positive association with plant cover.

The RDA ordination diagram (Figure 2) further indicates that no clear gradient existed in rehabilitation age with regard to microbiological activity; although the microbial activity in the topsoil of the youngest coal discard site (Bethal) was comparably lower than most of the older sites under rehabilitation. This could possibly be due to differences in management practices between the various sites.

Aon et al. (2001a) and Aon and Colaneri (2001) stated that plants may directly influence enzymatic activity, especially in the rhizosphere zone, primarily due to its high microbial activity because of the carbon input by plants, suggesting a plant-driven organisation of soil microbiology and biochemistry. Results observed during this study suggest that microbial activity and associated nutrient cycling is directly related to the percentage organic carbon in the soil, which is also related to the percentage ground and crown cover present on the sites investigated. These results suggest that the soil quality and fertility of the rehabilitated coal discard sites could eventually improve as long as vegetation growth and subsequent organic carbon input into the topsoil can be sustained without significant detrimental effects to the microbial ecosystem. Based on the results obtained during this and previous studies, a decrease in SOM content will result in a decrease in microbial activity and a reduction in nutrient circulation. (Aon et al., 2001a).

3.2. Reference sites

3.2.1. Physical and chemical characteristics

Results obtained for the physical and chemical characterisation of the soil samples from the three reference sites are summarised in Table 1. A PCA ordination diagram illustrating the relationship between the physical and chemical characteristics of the soil at the reference sites is presented in Figure 3. Based on these results, it is evident that the soil sampled at the reference sites varied markedly between sites based on physical and chemical characteristics.

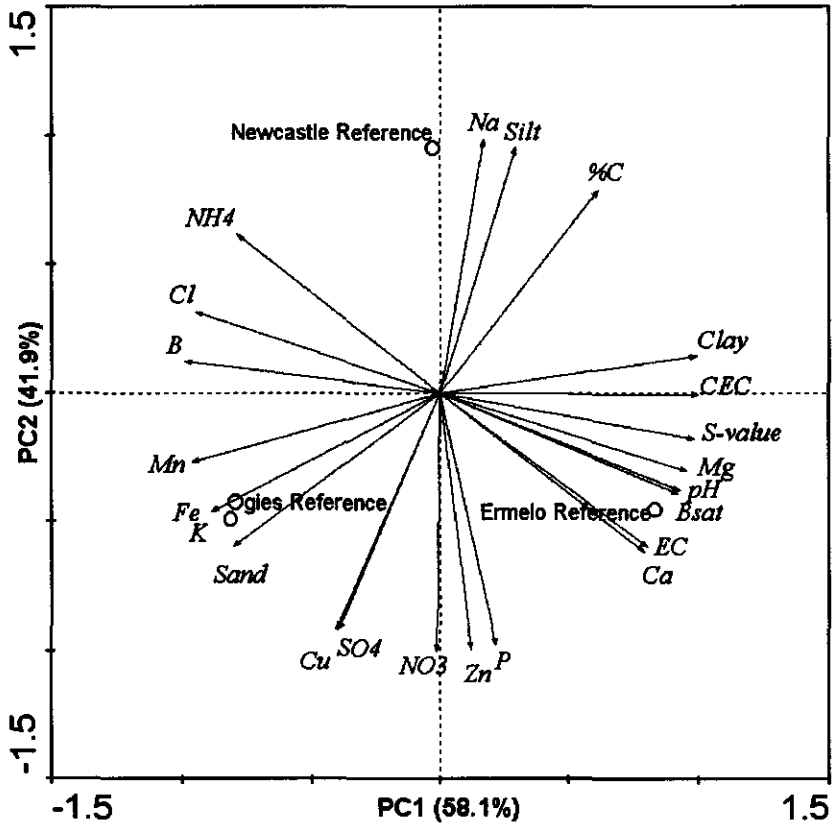

Figure 3. Principal Components Analysis (PCA) ordination diagram of the physical and chemical characteristics of the soil samples obtained from the three reference sites. The eigenvalues for the first two ordination axes of the PCA were 0.581 and 0.419, respectively. These two axes accounted for 100% of the total observed variance.

3.2.2. Vegetation coverage

The percentage ground and crown vegetation coverage as was evident during the time of soil sampling from the reference sites are summarised in Table 1. Only the Ogies reference site showed higher percentages of ground and crown cover than the seven coal discard sites.

3.2.3. Microbial counts

The CFUs obtained for the different media are summarised in Table 2. With the exception of the copiotrophic heterotrophic bacteria enumerated on R2A, statistical analysis of the microbial counts indicated no significant difference (p>0.05) between the various sites based on conventional microbial enumeration techniques (Table 2).

3.2.4. Enzymatic activities

The average activities of the enzymes assayed are presented in Table 2. According to the one-way ANOVA, the enzymatic activities assayed during this study showed an overall statistically significant difference among the reference sites at a probability level of 0.05 or less (Table 2).

An RDA ordination diagram illustrating the association between the dominant environmental variables, microbial enzymatic activities, microbial counts and percentage ground and crown cover of the reference sites is presented in Figure 4. The average values of the dominant soil chemical variables used in the RDA analysis are presented in Table 1.

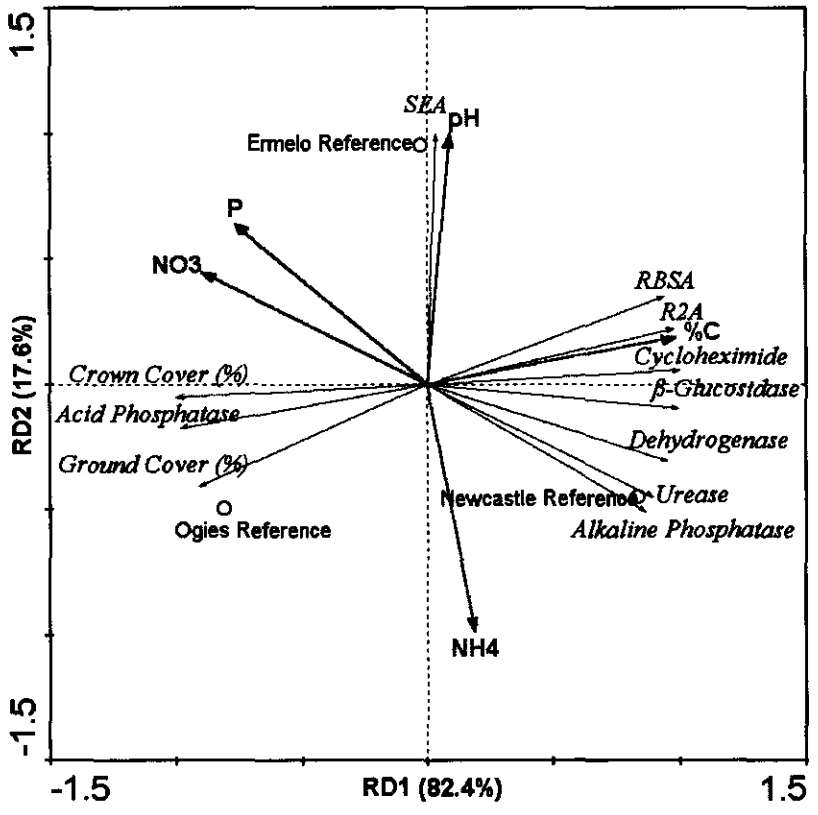

Figure 4. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables, soil microbiological enzymatic activities, bacterial and fungal colony forming units quantified on different media as well as the ground and crown vegetation coverage of the soil samples of the reference sites. Enzymatic activity, bacterial and fungal colony forming units and percentage ground and crown coverage of the vegetation are represented by solid vectors and the environmental physical / chemical parameters by dashed vectors. Eigenvalues for the first two axes are 0.824 and 0.176 respectively. Total observed variance of the first two canonical axes is 100%. The first canonical axis correlated strongly with percentage organic carbon (%C) (r^2 = 0.9827) and the second axis correlated with pH (r^2 = 0.9961). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis as well as the overall effect of the chosen environmental variables on the microbial enzymatic activities was not statistically significant (p = 1.000).

The results obtained indicate a strong positive association between the percentage carbon and the dehydrogenase, β-glucosidase, urease and alkaline phosphatase activities. Acid phosphatase activity was negatively associated with the percentage carbon and showed some positive association with the nitrate and phosphorus (Bray 1) concentrations. Urease

showed a weak association to the ammonium concentration and a strong negative association to the phosphorus and nitrate concentrations. Alkaline phosphatase activity was also negatively associated with phosphorus and nitrate. In contrast to the rehabilitation sites, no association was observed between pH and any of the enzymatic activities in the reference samples.

Dehydrogenase, β-glucosidase, urease and alkaline phosphatase activity was relatively high and showed a positive association with the total heterotrophic, bacterial copiotrophic and fungal counts. A negative association could be observed between the same plate counts and acid phosphatase. No association existed between any of the enzymes and oligotrophic bacterial counts.

Although the Ogies reference site had the highest percentage ground and crown vegetation cover, this site was characterised by the lowest microbiological and biochemical activities of the three reference sites studied.

4. CONCLUSIONS

According to Schoenholtz et al. (2000), it is often difficult to clearly separate soil functions into chemical, physical and biological processes because of the dynamic, interactive nature of these processes. Soil ecosystems have complex dynamics and no single property is satisfactory for studying microbial activity (Garcia et al., 2002). To obtain an accurate representation of the function and structure of soils, it is necessary to study the inter-relationship between physical, chemical, biochemical and biological properties (Trasar-Cepeda, 2000; Vepsäläinen et al., 2001). Measurement of only one or some of these properties will give only a partial evaluation of the state of the soil ecosystem. No single soil property is thus sufficient to evaluate the effect of anthropogenic or natural impacts on an ecosystem, because all methods are subject to limitations. It would therefore be most sensible to use a polyphasic approach – the combination of several types of techniques to assess soil quality.

The relevance of the results obtained in this study must be understood in the context of a search for a soil quality index as an indicator of sustainable management for the successful rehabilitation and revegetation of coal discard sites. Results obtained from the multifactorial analysis of microbiological and biochemical properties of soil illustrate that these properties are all interrelated and extensively influenced by the physical and chemical characteristics of soil being studied. Based on the results obtained during this study, it is evident that the absence or low percentage plant cover and associated lower levels of organic matter content of the topsoil covers have a significant negative impact on soil biochemical properties, including enzymatic activities and microbial population size. The lower microbial activity in the soils with degraded plant cover may result in decreased mineralisation rates and hence slower nutrient recycling. In the long term, these factors could result in decreased soil quality and fertility which could have a significant effect on the sustainable revegetation of the topsoils used as cover on the coal discard sites. Based on the results obtained during this study, it is evident that the characterisation of microbial enzymatic activities could serve as assessment criteria for the evaluation of the relative success of revegetation and subsequent rehabilitation of sites associated with discard mining material.

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CHAPTER 4

SOIL MICROBIAL COMMUNITY STRUCTURE BASED ON PHOSPHOLIPID FATTY ACID ANALYSIS AS ASSESSMENT CRITERIA FOR THE REHABILITATION OF COAL DISCARD SITES IN SOUTH AFRICA

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ABSTRACT

Assessment of soil microbial diversity is a sensitive indicator of ecological stress and restoration processes. During this study, the relationship between microbial community structure as determined by PLFA analysis, vegetation cover and physical and chemical characteristics of topsoil covers at seven coal discard rehabilitation sites were evaluated. A positive association was observed between the microbial biomass, vegetation cover and the %C, NH_4, NO_3 and the P concentrations. The various rehabilitation sites could be differentiated based on the microbial community structure. The Hendrina site was characterised by elevated levels of monounsaturated fatty acids, indicative of Gram-negative bacteria and high levels of physiological/nutritional stress. The Ermelo, Bethal, and Witbank 1 sites had elevated levels of polyunsaturated fatty acids and mid-branched saturated fatty acids, indicative of high levels of micro-eukaryotes, primarily fungi and Actinomycetes, respectively. These sites were also characterised by intermediate levels of Gram-negative bacteria, low levels of Gram-positive bacteria and low estimated viable microbial biomass. The Newcastle, Witbank 2 and Ogies sites had elevated levels of Gram-positive bacteria, intermediate levels of estimated viable biomass and intermediate to slightly lower levels of micro-eukaryotes (fungi), Actinomycetes and Gram-negative

bacteria. It is hypothesised that the microbial community structure of the Hendrina site is not sustainable when classified along an r-K gradient and that the high percentage of vegetation cover and high levels of estimated viable microbial biomass are an artificial reflection of the current management practices being employed at this site. Results obtained during this study indicate that the microbial community structure and the vegetation cover are significantly influenced by prevailing environmental physical and chemical and management characteristics and that the characterisation of microbial community structure by analysis of phospholipid fatty acids can be used as a rapid assessment criteria for the evaluation of the possible self-sustainability of rehabilitation of mine tailing and discard sites.

Keywords: Microbial community structure; Phospholipid fatty acids; Coal discard; Rehabilitation; Viable microbial biomass.

1. INTRODUCTION

Sustaining soil quality and fertility has become a major concern worldwide since soil is an essential natural resource (Pascual et al., 2000) that is intrinsically linked to overall environmental quality (Grayston et al., 2001; Marcote et al., 2001). Soil functions to produce plant biomass, maintain animal health and production, recycle nutrients, store carbon, partition rainfall, buffer anthropogenic acidity, remediate added animal and human wastes and regulate energy transformations (Doran and Zeiss, 2000; Schoenholtz et al., 2000). Under South African legislation the South African Environment Conservation Act (73/1989), requires developers to ecologically rehabilitate damaged environments, which calls for careful planning and implementation of sound silvicultural and ecological principles.

The coal discard wastes produced during coal mining in South Africa, are characterised by a high content of pyrite, which under moist oxidative conditions results in the formation of sulphuric acid due to both biological and chemical processes (Bell et al., 2001). This process is largely responsible for the formation of acid mine drainage and poor vegetation growth on mine discard sites. One control measure frequently applied for the containment of discard material is the application of a topsoil cover, which assists in the prevention of further oxidation and facilitates the subsequent revegetation of the site. The establishment of permanent self-sustaining vegetation on most mine waste sites in South Africa is, however, problematic in spite of the annual addition of large quantities of nitrogen and phosphorus fertilisers. This is to a large degree a function of both the quality and physical properties of the topsoil cover. Soil quality is dependent on a large number of chemical, physical and biological soil properties and its characterisation requires the selection of properties most sensitive to management practices and environmental stress. Biological properties of soil are responsive to small changes that occur in the soil, thereby providing immediate and accurate information on the changes in soil quality. Microbial communities are critical to soil quality because they play a fundamental role in plant establishment, soil formation, transformation of soil organic matter (SOM) (Mummey et al., 2002), and maintenance of biogeochemical cycles (Masciandaro and Ceccanti, 1999; Waldrop et al.,

2000). Poor management practices and other negative impacts on soil ecosystems result in the loss of functional groups of microorganisms and subsequent ecosystem modification or even collapse (Hawksworth, 1996). Assessment of soil microbiological properties and microbial diversity can therefore be used as sensitive indicators of both short and long-term changes (Hill et al., 2000) due to soil ecological stress or restoration processes, because they are responsive to small changes that occur in the soil environment (Badiane et al., 2001; Ibekwe et al., 2002).

The use of a culture-independent method (Hill et al., 2000), such as phospholipid fatty acid (PLFA) analysis circumvents many of the problems frequently associated with conventional culture dependent techniques and provides a more comprehensive view of the structure of complex microbial communities (Ibekwe and Kennedy, 1998; Pinkart et al., 1998). PLFAs are present in the cell membranes of all living microorganisms, which makes them valuable as signature molecules (Hill et al., 2000) and indicators of viable microbial biomass (Hill et al., 2000; Rütters et al., 2002). Recent studies suggest that PLFA profiles could be used as 'fingerprints' indicative of successful restoration of soil communities and as indicators of responses to management practices and changes in soil quality, and that changes in PLFA profiles could reflect past and present management practices (Ponder and Tadros, 2002; Steenwerth et al., 2003).

The lack of sufficient carbon inputs in most soils has led to the assumption that microorganisms exist at different levels of activity, with only a fraction of the microbial biomass being active (Ashman and Puri, 2002). This occurrence provides microorganisms with a strategy (r-K strategy) to ensure their continued existence, based on presumed differences in their ability to exploit resources and survive in different environments. Two groups are distinguished: r strategists prevail in unstable environments, while K strategists generally favour stable environments (Sarathchandra et al., 2001). Typically, r strategists compete better at low population densities because they have few competitive adaptations besides a rapid growth rate and could therefore be characteristic of populations initially colonising a habitat. On the other hand, K strategists reproduce slowly and depend on

physiological adaptations and the carrying capacity of the environment to survive (Atlas and Bartha, 1998; Ashman and Puri, 2002).

This study was undertaken to assess the influence of various physical and chemical parameters of the topsoil covers from seven coal discard sites currently under rehabilitation on the estimated viable microbial biomass and the microbial community structure as determined using PLFA analysis. The relationship between the environmental variables, the microbial community structure and the vegetation ground and crown coverage at the various sites was also investigated using multivariate statistical techniques. The objective of the study was to determine if the structural diversity of the soil microbial communities could possibly serve as assessment criteria for the successful rehabilitation and revegetation of ecologically disturbed areas, specifically coal discard sites.

2. MATERIALS AND METHODS

2.1. Site details

The study was conducted on seven already vegetated coal discard sites that are under rehabilitation and managed by Ingwe Closure Operations, Ingwe Mines, South Africa (site identities are presented in Table 1). The topsoil used as cover was excavated from adjacent borrow pits or stripped from the sites before mining. All the coal discard sites were vegetated with a grass seed mixture mostly dominated by the commercially available grasses *Eragrostis tef, Eragrostis curvula, Chloris gayana, Digitaria eriantha, Cynodon dactylon* and *Pennisetum clandestinum.* As a management practice, all dumps are constantly defoliated through mowing and/or cattle grazing, the latter being the preferred practice. All seven discard sites were also treated with variable amounts of lime, inorganic fertilisers and well-cured kraal manure at the onset of rehabilitation and during annual maintenance. As there were no suitable control sites available, random sites from the surrounding areas, representative of relatively natural veldt, were chosen as reference sites. These sites were located in areas adjacent to three of the coal discard sites (Table 1). All areas were situated within the summer rainfall area of South Africa and receive an average

annual rainfall between 700-800 mm. All the sites were located within the Grassland biome.

2.2. Sampling procedure

A random sampling design was used to obtain three composite samples per site ($n = 21$) of the topsoil from the seven coal discard sites (Alef and Nannipieri, 1995; Dick et al., 1996). Composite samples were also obtained from three reference sites ($n = 15$) in presumed undisturbed areas to represent natural grassland ecosystems. All samples were obtained during November 2002 in the same quadrates used for the assessment of vegetation growth. A soil auger was used to obtain volume samples and a minimum of 1 kg of soil per sampling area was obtained. Sampling was only conducted in the top 0-15 cm of the topsoil layer, since it has previously been reported that the number of bacteria and microbial activity decline with depth (Aon and Colaneri, 2001; Taylor et al., 2002). In addition, most of the dumps had an effective topsoil cover depth of only 10-15 cm and because of the compaction within the coal discard to prevent spontaneous combustion as well as unfavourable growth conditions, most of the plant root biomass was located within the 10-15 cm topsoil layer. Aseptic techniques were used as described by Dick et al. (1996). Each soil sample was sealed in plastic bags, frozen on site using dry ice and transported on dry ice to the laboratory, where it was stored at -86°C. Subsamples (± 200 g) of each sample were subsequently lyophilised before PLFA extraction, fractionation and analysis. The chemical and physical analyses of the topsoil of the respective sites were done as previously described (Van Rensburg et al., 1998). Twenty-four soil chemical and physical variables were analysed during this study.

2.3. Vegetation coverage

The ground and crown vegetation coverage of the rehabilitation and reference sites respectively, was visually estimated in three 1 m^2 quadrats randomly placed over a 50 m transect. The ground cover included all living and non-living organic material on the ground surface per area and the crown cover was regarded as the canopy cover spread of all

grass species over a fixed area. Both values are expressed as a percentage of surface area (1 m^2).

2.4. Phospholipid extraction, fractionation, and analysis

All glassware used for lipid analyses were washed with phosphate-free soap, rinsed five times with tap water and five times with distilled water, then air dried and heated in a muffle furnace at 450°C for a minimum of 4 h to remove any lipid contaminants. All solvents used were of the highest purity (Burdick and Jackson). Silicic acid and the internal standard, methyl nonadecanonate (C19:0) were obtained from Sigma Aldrich.

Total lipids were extracted from 5 g lyophilised soil according to a modified Bligh and Dyer procedure (White and Ringelberg, 1998). Silicic acid column chromatography (Guckert et al., 1985) was used to fractionate the total lipid extract into neutral lipids, glycolipids and polar lipids. The polar lipid fraction was transesterified to the fatty acid methyl esters (FAMEs) by mild alkaline methanolysis (Guckert et al., 1985). The FAMEs were analysed by capillary gas chromatography with flame ionisation detection on a Hewlett-Packard 6890 series II chromatograph fitted with a 60 m SPB-1 column (0.250 mm I.D., 0.250 μm film thickness). Definitive identification of peaks was undertaken using gas chromatography-mass spectrometry of selected samples using a Hewlett-Packard 6890 series II chromatograph interphased with a Hewlett-Packard 5973 mass selective detector. Methyl nonadecanonate (C19:0) was used as the internal standard and the PLFAs were expressed as equivalent peak responses to the internal standard. Standard fatty acid nomenclature was used (Ibekwe and Kennedy, 1998).

2.5. Statistical analysis

All samples were analysed in triplicate. PLFA profiles were analysed with STATISTICA 6 (StatSoft, Inc ©). Results of the signature lipid biomarker analyses were interpreted as described by Ringelberg et al. (1998). A three-tiered statistical approach was used where hypothesis testing was first made using analysis of variance (ANOVA) followed by the

application of multivariant analysis such as hierarchical clustering and principal components analysis (PCA) for the determination of sample relatedness. Redundancy analysis (RDA) was subsequently performed with the PLFA group-data, the percentage ground and crown vegetation coverage as species dependent variables, and the most significant soil variables as independent environmental factors. The most significant soil physical and chemical variables were selected through the forward selection procedure provided in CANOCO, thereby ensuring that only the most pertinent environmental gradients were investigated.

Factor analysis was also used as a data reduction method and in conjunction with hierarchical cluster analysis to assess changes in community structure. An ANOVA was performed on the factor scores and Tukey's Honest Significant Difference test (HSD) was used to identify significant differences between sites, with the within-experiment family-wise error rate set at $p = 0.05$.

Hierarchical cluster analyses were performed on the means from the transformed mol percent (mol%) PLFA using Ward's method. Factor analyses of biomass and arcsine square root transformed PLFA data were performed using both iterative and non-iterative extraction techniques (Peacock et al., 2001). Factor analysis was also used as a data reduction method and in conjunction with hierarchical cluster analyses to assess changes in community structure.

3. RESULTS AND DISCUSSION

3.1. Sites under rehabilitation

3.1.1. Physical and chemical characteristics of the various topsoil-covers

Results obtained for the physical and chemical characterisation of the topsoil samples from the various coal discard sites are summarised in Table 1. A PCA ordination diagram (Figure 1) indicated that the topsoil used as cover at the various coal discard sites varied

markedly between sites based on physical and chemical characteristics. This could have a significant impact on the structural diversity of the bacteria and fungi present within the various sites as well as on the rehabilitation and establishment of a self-sustaining vegetation cover at these sites.

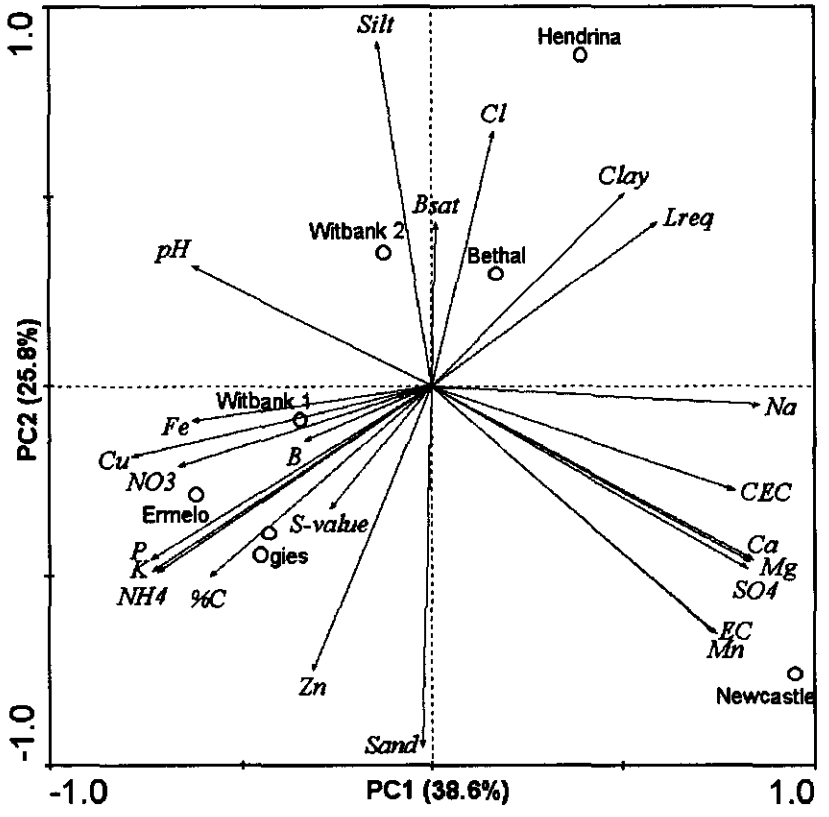

Figure 1. Principal Components Analysis (PCA) ordination diagram of the physical and chemical characteristics of the topsoils used as cover at the various coal discard sites. The eigenvalues for the first two ordination axes of the PCA were 0.386 and 0.258, respectively. These two axes accounted for 64.5% of the total observed variance.

Table 1. Physical and chemical properties of the topsoil covers obtained from the seven coal discard sites and three reference sites.

Properties	Rehabilitation sites							Reference sites		
	Newcastle	Ermelo	Hendrina	Bethal	Witbank 1	Witbank 2	Ogies	Newcastle	Ermelo	Ogies
Grid Reference	27°51'14"S 29°57'23"E	26°29'41"S 29°45'34"E	26°20'4"S 29°53'19"E	26°10'8"S 29°21'2"E	25°53'42"S 29°10'27"E	25°54'35"S 29°11'21"E	26°01'22"S 29°06'06"E	27°50'S 29°56'E	26°19'S 29°35'E	26°00'S 29°04'E
Rehabilitation age (years)	8	3	4	1	4	4	4	N/A	N/A	N/A
Ca ($mg\ kg^{-1}$)	49.30 ± 13.04	7.36 ± 0.27	3.73 ± 1.68	25.10 ± 9.72	2.92 ± 0.53	3.13 ± 0.27	5.65 ± 1.29	0.05 ± 0.02	0.22 ± 0.17	0.10 ± 0.02
Mg ($mg\ kg^{-1}$)	15.77 ± 3.40	2.08 ± 0.22	1.47 ± 0.64	7.89 ± 4.41	0.92 ± 0.18	0.98 ± 0.16	1.22 ± 0.49	0.19 ± 0.02	0.33 ± 0.15	0.15 ± 0.01
K ($mg\ kg^{-1}$)	6.29 ± 1.71	5.31 ± 0.78	25.08 ± 8.32	5.90 ± 1.96	4.92 ± 1.26	12.59 ± 6.51	24.59 ± 9.31	0.21 ± 0.01	0.18 ± 0.07	0.55 ± 0.14
Na ($mg\ kg^{-1}$)	2.08 ± 0.30	1.85 ± 0.12	1.21 ± 0.44	1.56 ± 0.44	0.93 ± 0.12	0.87 ± 0.27	1.21 ± 0.17	0.13 ± 0.07	0.05 ± 0.01	0.03 ± 0.00
SO_4 ($mg\ kg^{-1}$)	239.00 ± 72.57	28.27 ± 2.17	13.77 ± 4.72	122.04 ± 71.69	25.62 ± 12.21	26.34 ± 6.06	32.62 ± 15.90	0.16 ± 0.03	0.18 ± 0.06	0.19 ± 0.04
NO_3 ($mg\ kg^{-1}$)	2.45 ± 1.23	6.38 ± 1.30	16.19 ± 6.59	6.38 ± 5.15	1.31 ± 0.16	6.05 ± 3.50	11.45 ± 7.31	0.08 ± 0.04	0.24 ± 0.18	0.25 ± 0.03
NH_4 ($mg\ kg^{-1}$)	0.39 ± 0.07	0.26 ± 0.07	0.73 ± 0.23	0.30 ± 0.04	0.43 ± 0.09	0.64 ± 0.07	0.47 ± 0.21	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.00
Cl ($mg\ kg^{-1}$)	2.59 ± 0.18	12.75 ± 5.20	5.71 ± 1.96	5.35 ± 0.71	1.69 ± 0.32	4.01 ± 1.35	3.03 ± 1.03	0.30 ± 0.09	0.18 ± 0.08	0.33 ± 0.11
Fe ($mg\ kg^{-1}$)	33.44 ± 18.89	81.48 ± 16.06	5659.82 ± 2273.28	105.93 ± 89.03	5937.56 ± 2048.50	11956.04 ± 743.35	2126.28 ± 878.29	23.12 ± 1.64	21.52 ± 3.61	43.05 ± 13.26
Mn ($mg\ kg^{-1}$)	837.25 ± 480.20	16.45 ± 15.02	47.96 ± 14.59	5.25 ± 5.05	19.90 ± 5.57	75.32 ± 6.21	29.99 ± 6.24	2.56 ± 0.42	2.20 ± 0.90	3.49 ± 0.63
Cu ($mg\ kg^{-1}$)	0.96 ± 0.96	3.99 ± 3.99	31.29 ± 4.05	6.86 ± 1.94	8.14 ± 8.14	19.63 ± 8.16	8.62 ± 5.44	0.37 ± 0.22	0.88 ± 0.42	1.25 ± 0.30
Zn ($mg\ kg^{-1}$)	16.94 ± 9.69	6.74 ± 6.50	19.08 ± 4.42	5.59 ± 2.77	4.28 ± 3.79	21.88 ± 6.96	17.27 ± 0.57	0.95 ± 0.14	1.36 ± 0.76	1.31 ± 0.12
B ($mg\ kg^{-1}$)	35.36 ± 35.36	2.72 ± 2.72	0.00 ± 0.00	32.64 ± 16.32	130.54 ± 64.76	89.75 ± 63.38	127.83 ± 67.34	30.83 ± 7.24	22.50 ± 10.52	35.79 ± 2.28
P ($mg\ kg^{-1}$)	18.97 ± 7.53	5.60 ± 0.84	146.43 ± 65.07	18.23 ± 9.75	27.37 ± 7.27	38.13 ± 16.77	96.77 ± 17.27	7.01 ± 1.70	5.51 ± 0.69	17.98 ± 0.25
pH	5.51 ± 0.27	6.20 ± 0.26	7.03 ± 0.030	7.41 ± 0.44	7.15 ± 0.50	6.24 ± 0.41	6.90 ± 0.37	5.74 ± 0.30	6.66 ± 0.48	5.60 ± 0.05
Electrical Conductivity (EC) ($mS\ m^{-1}$)	117.33 ± 46.25	21.67 ± 4.10	29.00 ± 6.27	37.00 ± 4.36	26.33 ± 2.03	18.67 ± 6.23	35.00 ± 8.02	23.00 ± 3.27	29.75 ± 10.90	24.00 ± 3.61
Sand (%)	79.22 ± 5.53	39.80 ± 2.62	68.18 ± 2.53	58.70 ± 1.00	61.23 ± 1.62	68.12 ± 2.61	67.46 ± 0.91	55.57 ± 4.17	58.09 ± 11.12	80.73 ± 3.68
Silt (%)	8.46 ± 1.96	42.66 ± 1.76	23.42 ± 2.84	34.27 ± 1.54	26.27 ± 2.61	20.93 ± 2.92	25.25 ± 2.83	23.67 ± 4.48	14.04 ± 4.09	9.43 ± 2.27
Clay (%)	12.31 ± 3.81	17.55 ± 0.86	8.39 ± 0.51	7.02 ± 1.63	12.51 ± 1.10	10.95 ± 1.75	7.29 ± 2.26	20.76 ± 1.29	27.87 ± 7.48	9.84 ± 1.54
% Organic Carbon (%C)	0.69 ± 0.14	17.55 ± 1.91	2.20 ± 0.74	0.31 ± 0.16	0.32 ± 0.08	0.67 ± 0.01	2.72 ± 1.36	2.98 ± 0.77	2.44 ± 0.75	1.48 ± 0.43
Cation Exchange Capacity (CEC) $cmol(+).kg^{-1}$	24.34 ± 1.60	17.55 ± 1.92	9.34 ± 1.56	7.06 ± 1.77	4.19 ± 0.61	4.90 ± 1.01	9.92 ± 3.95	13.97 ± 3.29	22.35 ± 8.39	6.36 ± 0.95
S-value $cmol(+).kg^{-1}$	13.12 ± 0.17	12.80 ± 3.59	9.58 ± 2.81	8.46 ± 3.43	4.18 ± 1.07	3.23 ± 1.20	95.17 ± 14.20	8.76 ± 2.68	19.74 ± 8.06	3.50 ± 0.91
Base Saturation (Bsat) (%)	54.33 ± 3.07	70.13 ± 12.70	98.94 ± 15.35	112.29 ± 17.54	96.49 ± 13.94	61.28 ± 13.34	6.90 ± 0.37	56.43 ± 6.78	81.66 ± 11.51	53.34 ± 5.67
Lime requirement (Lreq) ($t.ha^{-1}$)	3.55 ± 2.21	8.05 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.62 ± 0.62	1.62 ± 1.04	0.67 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
% Soil Moisture	6.61 ± 0.35^{ac}	9.87 ± 2.13^{c}	6.84 ± 0.57^{ac}	4.35 ± 0.27^{ab}	1.68 ± 0.04^{b}	2.59 ± 0.29^{ab}	3.02 ± 0.75^{ab}	10.50 ± 1.45^{a}	9.26 ± 2.11^{a}	3.79 ± 1.31^{a}
% Ground Cover	77.42 ± 17.71^{abc}	63.21 ± 23.72^{a}	71.25 ± 19.10^{cd}	41.57 ± 15.67^{ab}	28.92 ± 12.30^{ab}	86.75 ± 16.59^{d}	60.91 ± 14.97^{bcd}	59.00 ± 11.00^{x}	59.00 ± 12.59^{x}	100.00 ± 0.00^{x}
% Crown Cover	56.29 ± 19.85^{b}	38.75 ± 12.36^{b}	80.13 ± 12.95^{b}	45.71 ± 15.97^{a}	42.50 ± 22.11^{a}	84.83 ± 14.27^{b}	65.91 ± 19.98^{ab}	67.00 ± 8.89^{xy}	52.50 ± 9.68^{x}	100.00 ± 0.00^{y}

[1] Values given are mean ± standard error

[2] Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

3.1.2. Vegetation coverage

The percentage ground and crown vegetation cover as was evident during the time of sampling of the topsoil from the various coal discard sites are summarised in Table 1. The highest percentage ground and crown cover was observed at the Hendrina and Witbank 2 sites. This could possibly be attributed to the elevated levels of phosphorus and ammonium in the topsoil covers of these sites (Table 1). The percentage ground and crown vegetation cover at the Bethal and Witbank 1 sites were significantly lower ($p<0.05$) than that of the other sites, primarily due to the dominance of large tufted grasses with a large crown cover and a smaller basal cover at this site. These sites were also characterised by very low percentages of organic material as well as a low percentage of stoloniferous grasses. The percentage crown cover at the Ermelo site was also significantly lower ($p<0.05$) than the other sites. This can be ascribed to excessive grazing of this site by cattle. A large quantity of organic matter was also present on the soil surface at the Ermelo site due to wastage during grazing activities. Reduced aboveground plant diversity as a result of tillage, overgrazing, pollutants and pesticides has been reported to decrease the microbial diversity in the soil ecosystem and to disturb its normal functioning. A degraded plant cover generally results in lower SOM content. This would imply a lower microbial activity due to the decreased availability of organic matter for decomposition (Aon et al., 2001; Garcia et al., 2002).

3.1.3. Phospholipid fatty acid analyses

Estimated viable biomass and statistical differences between the various soil samples using PLFA data is presented in Table 2. The biomass abundance in these samples as described by the PLFA concentration ranged from 6937.89 to 29 653.84 pmol per gram dry weight for the Hendrina and Bethal sites, respectively (Table 2). On average, the topsoil layers at Newcastle, Hendrina, Witbank 1, Witbank 2 and Ogies rehabilitation sites had significantly higher estimated biomass compared to Ermelo and Bethal ($p<0.05$) (Table 2). The Bethal coal discard site, which was the youngest coal discard site studied, contained the lowest estimated biomass, while Hendrina, which is 4 years old, contained the highest biomass.

This could possibly be ascribed to the higher nutrient availability at these sites (Table 1, Figure 1).

Phospholipid fatty acids can be grouped into major groups encompassing normal saturated fatty acid (Nsats), Mid-branched saturated fatty acids (MBsats), Terminally branched saturated fatty acids (TBsats), Branched monounsaturated fatty acids (Bmonos), Monounsaturated fatty acids (Monos) and Polyunsaturated fatty acids (Polys). The microbial community structure based on relative mol% fractions of the major phospholipid fatty acid groups is presented in Figure 2. The mol% fractions as well as statistical differences between the various sites on the basis of the major PLFA groups are summarised in Table 2.

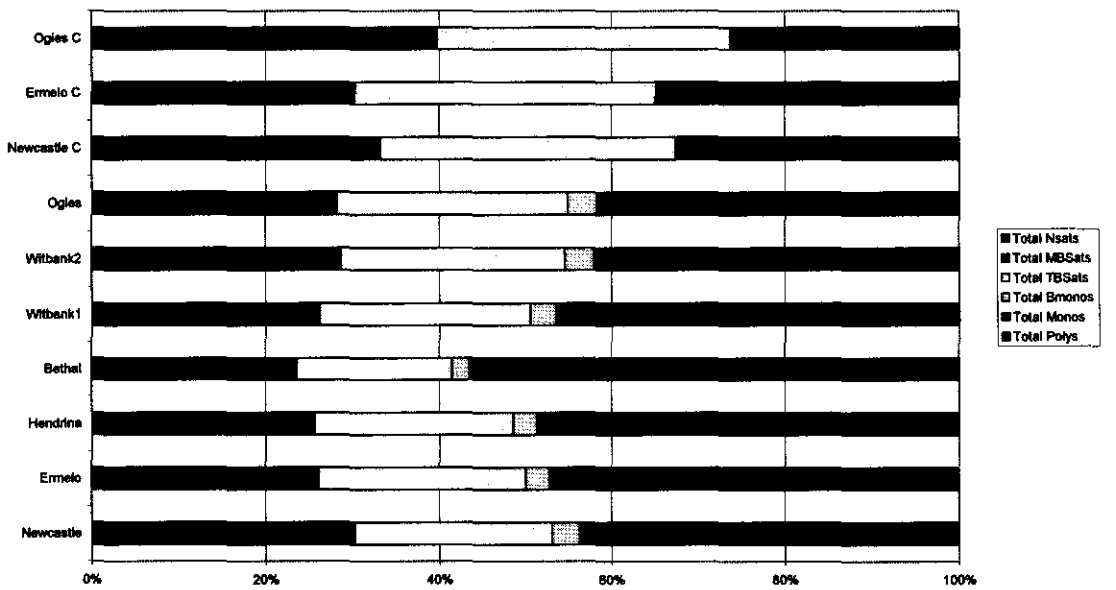

Figure 2. Microbial community structure based on the percentage fraction of the major phospholipid fatty acid groups.

Table 2. Percentage fractions of PLFA groups and ratios of specific lipid biomarkers from samples taken at the different coal discard and reference sites.

	Rehabilitation sites							Reference sites		
PLFA Major Group	**Newcastle**	**Ermelo**	**Hendrina**	**Bethal**	**Witbank 1**	**Witbank 2**	**Ogies**	**Newcastle**	**Ermelo**	**Ogies**
Normal saturated	27.85 ± 0.77^{e}	22.35 ± 1.35^{ab}	23.44 ± 0.63^{bc}	20.46 ± 0.28^{a}	23.69 ± 0.57^{bc}	26.01 ± 1.08^{de}	25.13 ± 0.63^{cd}	31.56 ± 2.37^{m}	30.80 ± 5.03^{m}	39.38 ± 3.41^{m}
Mid-chain branched saturated	2.32 ± 0.16^{ab}	3.08 ± 0.27^{c}	2.11 ± 0.09^{e}	2.98 ± 0.29^{bc}	2.59 ± 0.06^{abc}	2.61 ± 0.20^{abc}	2.46 ± 0.90^{abc}	3.72 ± 0.29^{m}	2.56 ± 0.65^{m}	1.20 ± 0.14^{m}
Terminally branched saturated	22.57 ± 13.28^{b}	24.09 ± 0.77^{bc}	22.84 ± 0.25^{b}	18.13 ± 0.64^{a}	25.25 ± 0.72^{bc}	25.47 ± 1.68^{bc}	26.98 ± 1.07^{c}	33.54 ± 1.3^{m}	33.90 ± 1.25^{m}	33.17 ± 0.98^{m}
Branched monounsaturated	3.24 ± 0.20^{bcd}	2.90 ± 0.20^{bc}	2.77 ± 0.08^{b}	2.06 ± 0.01^{a}	3.11 ± 0.10^{bcd}	3.35 ± 0.10^{cd}	3.47 ± 0.15^{d}	0.00 ± 0.00^{m}	0.00 ± 0.00^{m}	0.00 ± 0.00^{m}
Monounsaturated	33.51 ± 2.09^{a}	35.12 ± 0.40^{a}	41.82 ± 1.11^{c}	39.32 ± 0.17^{bc}	35.78 ± 0.82^{ab}	34.09 ± 0.45^{a}	35.95 ± 1.24^{ab}	26.08 ± 2.23^{m}	27.95 ± 3.99^{m}	21.64 ± 2.45^{m}
Polyunsaturated	10.40 ± 0.77^{bc}	12.05 ± 0.42^{c}	6.89 ± 0.63^{a}	17.05 ± 0.40^{d}	9.57 ± 0.65^{bc}	8.47 ± 0.83^{ab}	6.01 ± 0.85^{a}	3.98 ± 0.55^{m}	3.23 ± 0.56^{m}	3.08 ± 0.16^{m}
Total estimated biomass	$16277.30 \pm 5704.01^{ab}$	10315.14 ± 4745.73^{a}	$29850.56 \pm 9110.88^{ab}$	7108.01 ± 1692.12^{a}	$12043.97 \pm 4417.18^{ab}$	$15368.61 \pm 3357.34^{ab}$	$17719.66 \pm 2875.71^{ab}$	$49676.68 \pm 31515.69^{n}$	$39854.20 \pm 29537.01^{mn}$	$22503.79 \pm 8519.65^{mn}$
Ratios										
Fungal / Bacterial[2]	0.24 ± 0.06^{cd}	0.21 ± 0.10^{bcd}	0.12 ± 0.02^{abc}	0.32 ± 0.06^{d}	0.16 ± 0.04^{abc}	0.13 ± 0.07^{abc}	0.09 ± 0.02^{ab}	0.08 ± 0.04^{a}	0.04 ± 0.03^{m}	0.03 ± 0.00^{m}
Saturated / Unsaturated	1.45 ± 0.27^{abc}	1.33 ± 0.13^{ab}	1.09 ± 0.04^{a}	1.00 ± 0.07^{a}	1.29 ± 0.01^{ab}	1.45 ± 0.01^{abc}	1.41 ± 0.04^{abc}	2.41 ± 0.46^{op}	2.07 ± 0.36^{nop}	3.20 ± 0.82^{p}
16:1ω7t / 16:1ω7c	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.03 ± 0.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.02 ± 0.01^{a}	0.01 ± 0.02^{a}	0.10 ± 0.02^{m}	0.09 ± 0.03^{mn}	1.03 ± 0.14^{n}
cy17:0 / 16:1ω7c	0.52 ± 0.05^{a}	0.49 ± 0.05^{a}	0.46 ± 0.04^{a}	0.50 ± 0.07^{a}	0.57 ± 0.12^{a}	0.62 ± 0.07^{a}	0.66 ± 0.24^{a}	0.99 ± 0.17^{m}	0.96 ± 0.03^{m}	3.03 ± 1.80^{m}
18:1ω7t / 18:1ω7c	0.03 ± 0.04^{a}	0.01 ± 0.02^{a}	0.05 ± 0.02^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.04 ± 0.03^{a}	0.06 ± 0.01^{a}	0.17 ± 0.02^{m}	0.08 ± 0.01^{m}	0.29 ± 0.23^{m}
cy19:0 / 18:1ω7c	0.44 ± 0.37^{a}	0.36 ± 0.21^{a}	0.69 ± 0.38^{a}	0.37 ± 0.13^{a}	0.72 ± 0.28^{a}	0.68 ± 0.40^{a}	0.67 ± 0.72^{a}	1.71 ± 1.26^{m}	1.20 ± 1.60^{m}	5.51 ± 1.60^{m}

[1] Sites with the same combination of superscript alphabetic letters indicate no significant differences among sites.

[2] Calculated according to Frostegaard and Bååth (1996).

Normal saturated fatty acids occur in most microorganisms and are therefore considered ambiguous. Terminally branched saturated fatty acids are generally considered indicative of Gram-positive bacteria (Zelles, 1999). Based on the results summarised in Table 2, it is evident that the lowest concentration of Gram-positive organisms was present at Bethal, while the highest concentration was present at the Ogies site. Monounsaturated fatty acids are generally considered indicative of Gram-negative bacteria (Ratledge and Wilkinson, 1988). It is thus evident that Hendrina had the highest relative concentration of Gram-negative bacteria. White et al. (1996) reported that polyunsaturated PLFAs are found almost exclusively in eukaryotes. The highest concentration of polyunsaturated fatty acids was observed at Bethal, while the lowest concentration was present at the Ogies site. This indicates a higher occurrence of fungi in the Bethal site than the other sites.

Frostegård and Bååth (1996) suggested the use of the sum of PLFAs considered to be predominantly of bacterial origin (i15:0, a15:0, i16:0, 16:1ω9, 16:1ω7t, i17:0, a17:0, 17:0, cy 17:0, 18:1ω7 and cy 19:0) as an index of the bacterial biomass (BactPLFA). The quantity of 18:2ω6 was used as an indicator of fungal biomass (FungPLFA), since it is mainly of fungal origin (Merilä et al., 2002). According to Bailey et al. (2002), the fungal/bacterial (F:B) ratios obtained from PLFA analysis are similar to those obtained by selective inhibition of substrate-induced respiration, with a F:B activity ratio of 1.0 indicating equal contribution of fungi and bacteria to the microbiological activity in the soil sample. When using PLFA analysis to determine F:B ratios, the ratio is usually <1.0 since the saturated fatty acids included in the prokaryotic lipids are ubiquitous and found in most organisms (Bailey, et al., 2002). The results obtained in this study indicate significant differences between the rehabilitation sites based on F:B ratios (Table 2). All the discard sites investigated showed F:B ratios of <1.0, with the highest ratio observed at the Bethal site, thus corresponding with the observation that this site had the highest concentration of polyunsaturated fatty acids.

During induced stationary-phase growth conditions, cyclopropane 17:0 and 19:0 PLFAs have been reported to accumulate in Gram-negative heterotrophic bacteria (Steger et al.,

2003). Exponentially growing cells have been reported to have cyclopropane PLFA/monoenoic PLFA ratios of <0.05 and an increase in these cyclopropane PLFA/monoenoic 16:1ω7c or 18:1ω7c PLFA precursors, to be indicative of possible nutritional stress (ratios >0.1) (White et al., 1996). All sites analysed during this study had cyclopropane PLFA/monoenoic PLFA ratios >0.1 (Table 2), suggesting that the microbial communities are under possible nutritional stress and that the majority of the cells are possibly experiencing nutritional stress or are in the stationary growth phase.

Ratios of the *trans/cis* isomers of the monounsaturated PLFAs 16:1ω7 and 18:1ω7 have been reported to be indicative of possible environmental stress or starvation in microorganisms (Mandelbaum et al., 1997). An increase in the *trans/cis* ratios of these isomers to >0,1 are regarded to be indicative of possible toxicities or starvation whereas healthy organisms have a ratio of <0,05. Using *cis/trans* isomeration, bacteria can adapt quickly to toxic concentrations of organic substrates and thereby stabilise their membranes and thus retain their intracellular physiological balance (Guckert et al., 1991). With the exception of Hendrina and Ogies, all of the rehabilitation sites evaluated during this study, had 18:1ω7t/18:1ω7c ratios of <0.05, indicative of low stress environments. Although, both Hendrina and Ogies were characterised as having the highest estimated viable biomass, these sites had the highest *trans/cis* ratios (Table 2) indicating stimulated growth under stressful conditions. It is possible that this growth could be attributed to r-strategic microorganisms, since they favour unstable environments (Sarathchandra et al., 2001).

An RDA triplot illustrating the association between the dominant environmental variables, microbial phospholipid fatty acid profiles and percentage ground and crown cover is presented in Figure 3.

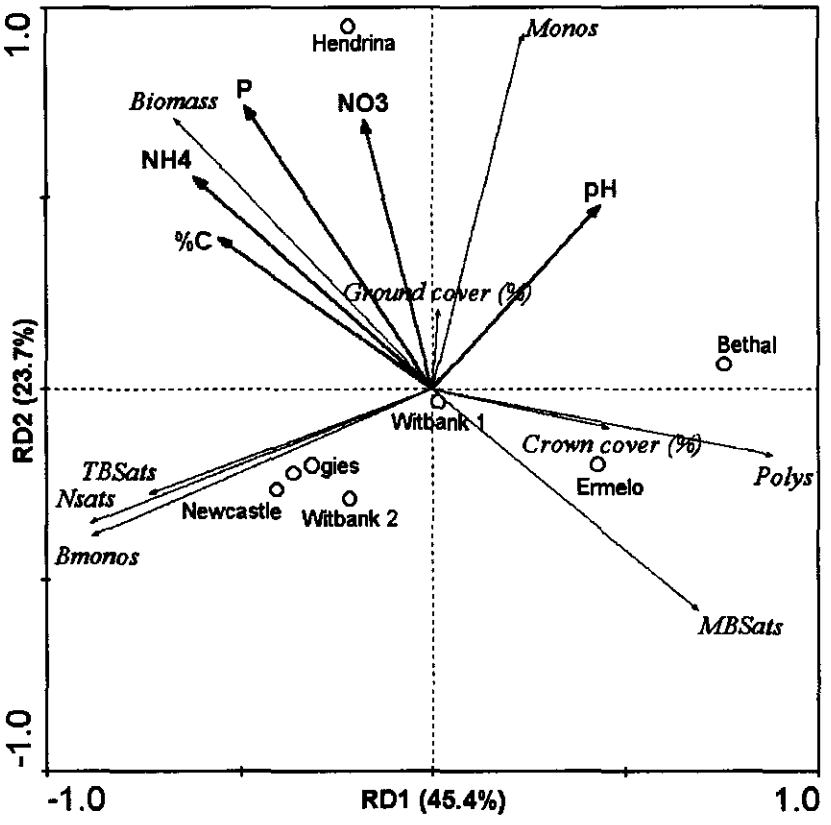

Figure 3. Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dominant environmental variables, phospholipid fatty acid profiles as well as the ground and crown coverage vegetation of the topsoil covers of the various coal discard sites. Phospholipid fatty acid groups and percentage ground and crown vegetation coverage are represented by solid vectors and the environmental physical / chemical parameters by dashed vectors. Eigenvalues for the first three axes were 0.535, 0.248 and 0.099, respectively. Total observed variance of the first two canonical axes was 81.9 %. The first canonical axis correlated strongly with NH_4 (r^2 = -0.6896) and percentage organic carbon r^2 = -0.6230). The second axis correlated with NO_3 (r^2 = 0.8027) and P (r^2 = 0.8016) and the third axis (not shown) correlated with pH (r^2 = -0.7314). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis was not statistically significant (p = 0.17), although the overall effect of the chosen environmental variables on the phospholipid fatty acid groups was statistically significant (p = 0.020). Key to major PLFA groups: Nsats (normal saturated), MBSats (mid-chain branched), TBSats (terminally branched saturated), Bmonos (branched monounsaturated), Monos (monounsaturated), Polys (polyunsaturated).

The average values of the dominant soil chemical variables used in the RDA analysis are summarised in Table 1. Based on the results obtained, it is evident that, estimated viable

biomass was strongly associated with the percentage organic carbon (%C), ammonium (NH_4), phosphorus (P) and nitrate (NO_3) in the topsoil covers. The sites investigated during this study could also be grouped by hierarchical clustering analysis of the major phospholipid fatty acid groups (Figure 4). The dendrogram obtained highlighted the grouping of the different sites. Two major clusters could be identified. Hendrina was the only site in cluster I, while all other sites grouped in cluster II. This cluster (II) could be further subdivided into two subclusters. Newcastle, Witbank 2 and Ogies grouped together in sub-cluster III. Ermelo, Bethal and Witbank 1 grouped together in sub-cluster IV.

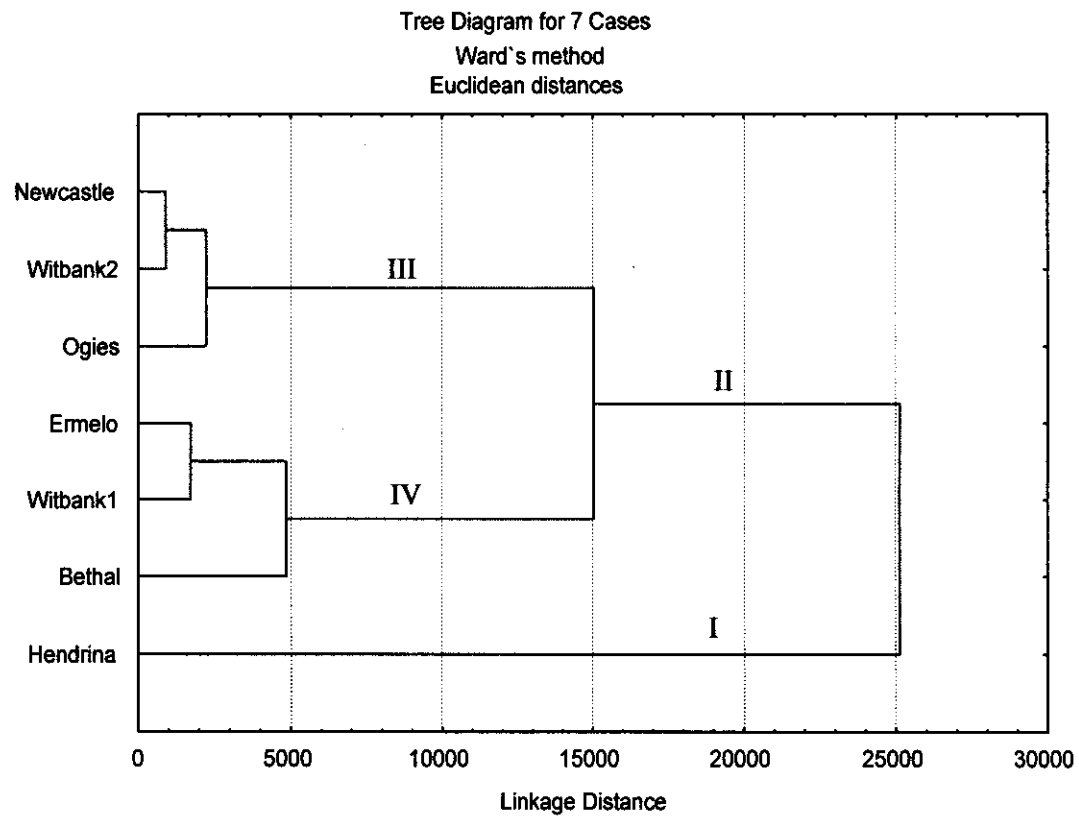

Figure 4. Dendrogram illustrating the clustering of samples based on phospholipid fatty acid profiles in the topsoil covers used at the various coal discard sites. Hierarchical cluster analysis was performed using Ward's clustering algorithm.

The Newcastle, Witbank 2 and Ogies sites grouped in the lower left hand quadrant of the first ordination axis (Figure 3) and clustered together in sub-cluster III (Figure 4). These sites were all characterised by elevated ratios of saturated to unsaturated phospholipids,

which could be indicative of change in the fluidity of the cell membranes of the microorganisms present (Keift et al., 1994). In comparison to the other sites, these sites were characterised by elevated levels of Gram-positive bacteria, as well as elevated cyclopropane 17/16:1ω7c ratios. The latter suggests that the microbial communities at these sites are possibly under nutritional stress.

Rehabilitation sites to the left of the first ordination axis (Newcastle, Hendrina, Witbank 2 and Ogies) (Figure 3) had elevated levels of soil microbial biomass as well as percentage ground and crown cover when compared to the sites (Ermelo, Bethal and Witbank 1) (sub-cluster IV, Figure 4) located to the right of this axis. This is probably related to the high organic carbon content, as well as the elevated levels of ammonium, nitrate and phosphorus at these sites. The highest level of viable soil microbial biomass was observed in the topsoil cover obtained from the Hendrina coal discard site. This observation may be a result of the management practices maintained at these sites, during which large amounts of fertiliser and organic material were incorporated into the topsoil cover. This phenomenon may only be a temporary observation, since the high levels of estimated biomass would only be sustained as long as the nutrient concentrations remain in excess. It is also interesting to note that despite the highest estimated biomass levels, the Hendrina site was also characterised by the highest levels of stress and less-optimal growth conditions, as indicated by the higher *trans/cis* ratios as well as the higher cyclopropane 19/18:1w7c ratios. The low estimated viable biomass as observed at the Ermelo, Bethal and Witbank 1 sites could be indicative of low overall microbial activity at these sites. These sites were also characterised as being the youngest sites evaluated during this study, as well as by the lowest organic carbon content and percentage ground and crown vegetation coverage (Table 1). Consequently, soil quality and fertility of the topsoil covers at these sites are lower because of lower microbial activity due to the deficit of biodegradable organic matter and limited nutrient recycling. This could have a direct impact on the self-sustainability of the vegetation cover at these sites.

Results obtained from the RDA triplot (Figure 3) further indicate that no clear gradient existed in rehabilitation age with regard to viable microbial biomass, although the

estimated microbial biomass in the topsoil of the youngest coal discard site (Bethal) was comparably lower than most of the older sites under rehabilitation. This could be due to differences in management practices between the various sites. Results observed during this study and other studies suggest that microbial activity and associated nutrient cycling is related to the percentage organic carbon in the soil, which is also related to the percentage ground and crown vegetation cover present on the sites investigated. This suggests that soil quality and fertility at these rehabilitation sites could eventually improve as long as vegetation growth and subsequent organic carbon input into the topsoil could be sustained without significant detrimental effects to the microbial ecosystem. One of the major driving components currently employed for the generation of the required organic biomass is nitrogen fertiliser application. A balance, however, has to be maintained to ensure that the application of fertilisers (including quantity and frequency of application) do not exceed the functional ability of the soil to retain and transform the nutrients. Once sufficient vegetation cover has been established, the ecosystem could become self-sustainable and maintain soil quality.

3.2 Reference sites

3.2.1. Physical and chemical characteristics

Physical and chemical characterisation of the soil samples obtained from the three reference sites are summarised in Table 1. A PCA ordination diagram indicated that the soil physical and chemical characteristics sampled at the reference sites varied markedly between sites (Figure 5).

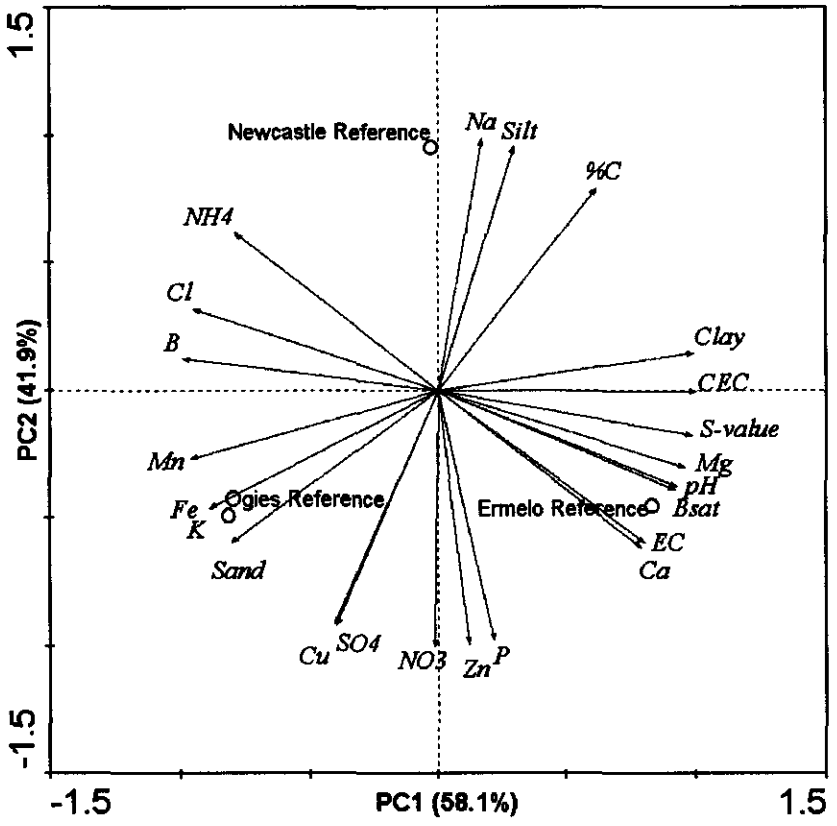

Figure 5. Principal Components Analysis (PCA) ordination diagram of the physical and chemical characteristics of the soil samples obtained from the three reference sites. The eigenvalues for the first two ordination axes of the PCA were 0.581 and 0.419, respectively. These two axes accounted for 100% of the total observed variance.

3.2.2. Vegetation coverage

The percentage ground and vegetation crown cover as was evident during the time of soil sampling from the reference sites are summarised in Table 1. Only the Ogies reference site showed significantly higher percentages of ground and crown vegetation cover than the seven coal discard sites.

3.2.3. Phospholipid fatty acid analyses

Estimated biomass as determined for the reference soil samples using PLFA data is presented in Table 2. The viable biomass abundance in these samples as described by the PLFA concentration ranged from 49676.68 to 22503.79 pmol per gram dry weight. On average, the topsoil layers at the rehabilitation sites had significantly higher biomass values compared to the reference sites.

The mol% fractions as well as statistical differences between the various sites on the basis of the major PLFA groups are summarised in Table 2. There was no significant difference between the three reference sites based on major PLFA groups (p>0.05). Furthermore, it is evident that the average concentration of Gram-positive organisms present at the reference sites was significantly higher when compared to the rehabilitation sites. The average levels of Gram-negative bacteria and fungi were significantly lower at the reference sites in comparison to the rehabilitation sites.

The results obtained in this study indicate lower fungal/bacterial (F:B) ratios for the three reference sites compared to the seven rehabilitation sites investigated (Table 2). These results are in contrast with results obtained from a study by Bardgett and McAlister (1999), that suggest that the ratio of F:B PLFA are higher in native soils or soils that are unimproved, in contrast to soils that have been managed and show a lower ratio of F:B PLFA. (Bardgett and McAlister, 1999). The reason for the difference in results obtained from this study could be attributed to the annual addition of well-cured kraal-manure which would result in the addition of fungi to the system.

All reference sites analysed during this study had cyclopropane PLFA/monenoic PLFA ratios relatively higher than that of the rehabilitation sites. The ratio of *trans/cis* isomers of the monounsaturated PLFAs C16:1ω7 and 18:1ω7 was also higher (Table 2).

An RDA ordination diagram illustrating the association between the dominant environmental variables, microbial phospholipid fatty acid profiles and percentage

ground and crown vegetation cover determined at the reference sites, is presented in Figure 6.

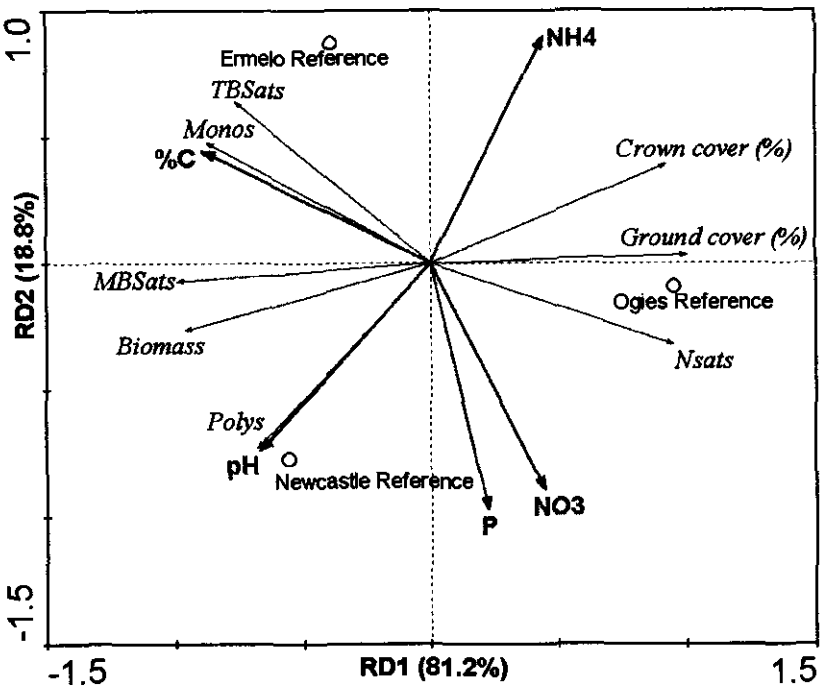

Figure 6. Redundancy Analysis (RDA) ordination diagram illustrating the association between the dominant environmental variables, phospholipid fatty acid profiles as well as the ground and crown vegetation coverage of the soil samples from the reference sites. Phospholipid fatty acid groups and percentage ground and crown vegetation coverage are represented by solid vectors and the environmental physical / chemical parameters by dashed vectors. Eigenvalues for the first two axes are 0.812 and 0.188, respectively. Total observed variance of the first two canonical axes is 100%. The first canonical axis correlated strongly with percentage organic carbon (%C) (r^2 = -0.8948) and the second axis correlated with P (r^2 = -0.9742). According to a Monte Carlo Permutation test conducted with 499 permutations the first canonical axis as well as the overall effect of the chosen environmental variables on the microbial phospholipid fatty acid profiles was not statistically significant (p = 1.000). Key to major PLFA groups: Nsats (normal saturated), MBSats (mid-chain branched), TBSats (terminally branched saturated), Bmonos (branched monounsaturated), Monos (monounsaturated), Polys (polyunsaturated).

The average values of the dominant soil chemical variables used in the RDA analysis are summarised in Table 1. Based on the results obtained, it is evident the percentage organic carbon showed a positive association to estimated viable biomass and all the major PLFA

groups, except normal saturated fatty acids, which showed a strong negative association. The soil pH showed a strong positive association with polyunsaturated fatty acids as well as estimated viable biomass. The ammonium and phosphorus concentrations did not associate with any of the major PLFA groups. Nitrate concentration had a slight positive association with normal saturated fatty acids and a negative association with terminally branched saturated fatty acids and monounsaturated fatty acids. Only the normal saturated fatty acids showed a positive association to the percentage ground and crown vegetation cover, with all other major PLFA showing different degrees of negative association.

The Ogies Reference site was the only reference site with a high percentage ground and crown vegetation cover and high normal saturated fatty acids. This site had the lowest concentrations of all other major PLFA groups. The Newcastle reference site showed the highest estimated viable biomass and occurrence of polyunsaturated fatty acids even though it had lower percentage vegetation cover. The Ermelo reference site, which also had a relatively low percentage vegetation cover, showed a high amount or terminally branched saturated and monounsaturated fatty acids.

4. CONCLUSIONS

During this study, it was observed that PLFA analyses were sufficiently sensitive to detect differences in the community structure in the different rehabilitation and reference sites. When analysed in relation to environmental variables, including physical and chemical characteristics of the topsoil covers as well as the percentage ground and crown vegetation cover, significant results could be obtained. Based on the results obtained during this study, the Hendrina rehabilitation site was characterised by elevated levels of monounsaturated fatty acids, which is considered indicative of Gram-negative bacteria. The Newcastle, Witbank 2 and Ogies rehabilitation sites were characterised by elevated levels of branched-chain fatty acids which are considered to be indicative of Gram-positive bacteria, whereas the Ermelo, Bethal and Witbank 1 sites were characterised by elevated levels of polyunsaturated fatty acids, which is indicative of micro-eukaryotes,

primarily fungi. Microorganisms have evolved strategies that enable them to successfully survive and maintain themselves within communities and can be classified along an r-K gradient. The r strategists rely upon high reproductive rates for survival within a community, whereas the K strategists depend upon physiological adaptations to the environmental resources. When resources become scarce, r strategists experience rapid reduction, whereas K strategists tend to be successful in resource-limited situations. Many Gram-negative microorganisms are considered r strategists. It can thus be concluded that the elevated levels of Gram-negative bacteria in the Hendrina site could possibly be the response of r-strategists to the presence of the excessive nutrients. It is, however, evident that this condition is not sustainable, and that the microbial community within this site would experience a significant reduction in estimated viable biomass once the resources (%C, N and P) in this site become depleted. In contrast, the Ermelo, Bethal, and Witbank 1 and sites were characterised by elevated levels of micro-eukaryotes, primarily fungi and Actinomycetes and decreased levels of Gram-positive and Gram-negative bacteria. The Ermelo and Bethal rehabilitation sites were also the youngest of the sites evaluated during this study. On the basis of the delicate balance between r and K strategists as required to maintain ecosystem functionality, it may be concluded that the Newcastle, Witbank 2 and Ogies rehabilitation sites were characterised as having suitable biological properties to facilitate the establishment of self-sustainable vegetation cover. This, however, would have to be confirmed by both spatial and temporal monitoring of microbial activity, function and structure.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

1. BACKGROUND

Mining is one of a number of anthropogenic activities that adversely affect soil quality and fertility. In South Africa, specifically, mining activities are the biggest contributor to the solid waste stream (81%) and the natural grassland biome of the country is fragmented by an abundance of mine tailings and discard sites (Van Wyk, 2002). Even though mining in South Africa provides a vast contribution to the economy, with benefits to all aspects of society (Mining Review Africa, 2003), the extensive adverse effects on the biophysical, social and economic environments cannot be ignored (Milton, 2001; Mummey et al., 2002). Soil is a vital natural resource that is non-renewable on a human time-scale (Tate, 2000) and intrinsically linked to water and atmospheric quality and ultimately, to overall environmental sustainability (Marcote et al., 2001). In view of the considerable losses that have occurred in terms of soil quality in South Africa (DEAT, 1999) and other countries worldwide (Arshad and Martin, 2002), the urgency of finding rehabilitative measures to counteract the depletion of natural resources and environmental degradation has been recognised. Although mines are expected to provide for and apply rehabilitation measures before closure permits are granted (Milton, 2001), the restoration of disturbed mining areas is a complicated and multifaceted process. Past rehabilitative management strategies that relied extensively on the restoration of disturbed areas by application of agronomic approaches proved unsuccessful in the arid areas of southern Africa (Milton, 2001). However, it seems that vegetative stabilisation is the most successful answer to achieving sustainable rehabilitation of mine discard sites (Carroll et al., 2000) and since soil is the growth medium for all vegetation, it is important to find suitable methods to assess and improve the quality of this growth medium. Research should thus be focused on a more

integrated approach which takes the physical, chemical and biological properties and their interactions into account.

2. GENERAL DISCUSSION

The dynamic, interactive nature of soil physical, chemical and biological processes makes separation and independent assessment of such properties impossible. Soil ecosystems have complex dynamics and no single property is satisfactory for assessing soil quality or making predictions regarding the sustainability of these ecosystems. To obtain an accurate representation of the function and structure of soils and the effect of anthropogenic activities on the soil environment, it is necessary to study the inter-relationship between physical, chemical, biochemical and biological properties. It is therefore essential that a polyphasic approach be used with the combination of different assays to assess several types of soil properties and the use of different techniques to assess the same property until the best method is established. With the application of suitable assessment techniques and management strategies, such as those discussed in this and similar studies, it should still be possible to improve soil quality and fertility although complete restoration would most likely never be achieved.

The use of microbiological and biochemical properties of soil, such as enzymatic activities and microbial community structure, have often been proposed as early and sensitive indicators of soil ecological stress or restoration processes (Bandick and Dick, 1999; Badiane et al., 2001; Ibekwe et al., 2002). These properties are significantly more responsive to small changes that occur in soil than the physical and chemical properties that have been used traditionally (Pascual et al., 2000) and are valuable for monitoring trends in soil quality, such as during rehabilitation processes.

The coal discard waste produced during coal mining in South Africa, is characterised by a high content of pyrite, which under high moisture and oxidation conditions results in the formation of sulphuric acid due to both biological and chemical processes (Bell et al., 2001). One control measure frequently applied for the containment of discard material is

the application of a topsoil cover, which assists in the prevention of further oxidation and facilitates the subsequent revegetation of the site. The topsoil used as cover on the discard sites are excavated from adjacent borrow pits or stripped from the sites before mining activity commences. During this procedure soil horizons are mixed, which inevitably results in significant changes in the physical, chemical and biological aspects of the soil quality, including soil microbial community function and structure. The observed variation in physical and chemical characteristics obtained for the topsoil samples from the seven rehabilitation sites could significantly influence the enzymatic activity of the microorganisms and in turn impact on the nutrient cycling within these ecosystems. Such an impact would directly affect the sustainability of the revegetation and completion of the rehabilitation process at the various sites because of ecosystem modification. A low percentage of vegetation cover generally results in lower soil organic matter (SOM) content, resulting in lower microbial activity due to the decreased availability of organic matter for decomposition (Garcia et al., 2002).

Although significant differences ($p<0.05$) existed between the three reference sites based on conventional microbiological enumeration techniques, no significant difference ($p>0.05$) could be observed between the various rehabilitation sites based on the same techniques (Table 2, Chapter 3). This confirms that the use of conventional microbiological techniques that rely on the culturability of the microorganisms is often insensitive and provides little insight into the nutritional/environmental status *in situ* (White et al., 1996). Thus, it would prove more useful to apply alternative techniques such as enzymatic assays, phospholipid fatty acid (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE) and substrate induced respiration (SIR).

During this study, the relationship between the soil physico-chemical characteristics and the microbiological properties, as assayed using conventional microbiological methods, enzymatic activity and analysis of signature lipid biomarkers and vegetation variables was investigated using multivariate statistical analysis, including Principal Components Analysis (PCA) and Redundancy Analysis (RDA). According to the one-way ANOVA, the potential enzymatic activities assayed during this study showed an overall statistically

significant difference among the reference sites at a probability level of 0.05 or less (Table 2, Chapter 3). The analysis of the topsoil samples from the seven rehabilitation sites showed that ammonium (NH_4), nitrate (NO_3), phosphorous (P), pH and organic carbon content (%C) had the most statistically significant influence on the percentage vegetation cover, microbial activity (Figure 2, Chapter 3) and microbial community structure (Figure 3, Chapter 4). The acid phosphatase activity assayed at the reference sites was negatively associated with organic carbon content and showed some positive association with nitrate and phosphorus (Bray 1) concentrations. All other enzymatic activities assayed at the reference sites displayed a positive association with the organic carbon content of the soil. With the exception of alkaline phosphatase, a positive association was observed between all enzymatic activities and the nitrate and phosphorus concentrations at the rehabilitation sites. Alkaline phosphatase activity was also negatively associated with phosphorus and nitrate at the reference sites. The ammonium concentration assayed at the rehabilitation sites had a weak association with all the enzymes studied, except alkaline phosphatase, with which it showed a strong negative association. A negative association was also observed between pH and all enzymatic activities assayed at the rehabiliation sites. In contrast to the rehabilitation sites, no association was observed between pH and any of the enzymatic activities in the reference samples. Furthermore, at the rehabilitation sites dehydrogenase activity was high and positively associated with oligotrophic bacterial and fungal counts, as was also the case with alkaline phosphatase activity. Urease was observed to associate negatively with copiotrophic bacterial counts and positively with fungal counts, confirming the results reported by Aon et al. (2001) (Figure 2, Chapter 3)

Redundancy analysis (RDA) was also used to determine the relationship between microbial community structure, vegetation cover and physico-chemical characteristics of topsoil covers of the seven coal discard rehabilitation sites and soil samples from the reference sites (Figure 6, Chapter 4). The microbial community structure was characterised by analysis of signature lipid biomarkers, specifically the analysis of PLFAs. On average, the topsoil layers at the rehabilitation sites had significantly higher biomass values compared to the reference sites (Table 2, Chapter 4). A positive association existed between the estimated microbial biomass, vegetation cover and the organic carbon content, ammonium,

nitrate and the phosphorous concentrations, for the rehabilitation sites. The estimated biomass for the reference sites showed a positive association with the organic carbon content and pH, no association with phosphorus, ammonium or nitrate and a negative association with vegetation cover.

A clear differentiation could also be observed between the various rehabilitation sites based on the microbial community structure (Figure 2, Chapter 4). The fungal/bacterial (F:B) ratios obtained in this study were lower for the three reference sites compared to the 7 rehabilitation sites investigated (Table 2, Chapter 4). This could possibly be attributed to the annual addition of well-cured kraal-manure, which would result in the addition of fungi to the system. In contrast to the other rehabilitation sites, the Hendrina site was characterised by elevated levels of monounsaturated fatty acids, indicative of Gram-negative bacteria, and high levels of physiological/nutritional stress, based on cyclopropane PLFA/monenoic PLFA ratios (White et al., 1996) and ratios of the *trans/cis* isomers of the monounsaturated PLFAs C16:1ω7 and 18:1ω7 (Mandelbaum et al., 1997) (Chapter 4). These results suggest that the microbial community at this site is possibly under nutritional stress and that the majority of the cells might be experiencing nutritional stress or are in the stationary growth phase. The Ermelo, Bethal and Witbank 1 rehabilitation sites were all characterised by elevated levels of polyunsaturated fatty acids and mid-branched saturated fatty acids, indicative of high levels of micro-eukaryotes, primarily fungi and Actinomycetes. These sites were also characterised by intermediate levels of Gram-negative bacteria, low levels of Gram-positive bacteria and low estimated microbial biomass. The Newcastle, Witbank 2 and Ogies sites had elevated levels of Gram-positive bacteria, intermediate levels of estimated biomass and intermediate to slightly decreased concentrations of micro-eukaryotes (fungi), Actinomycetes and Gram-negative bacteria. In general, the average levels of monounsaturated PLFAs and polyunsaturated PLFAs, indicative of Gram-negative bacteria and fungi respectively, were significantly lower at the reference sites in comparison to the rehabilitation sites (Table 2, Chapter 4). Furthermore, the percentage organic carbon showed a positive association to estimated biomass and all the major PLFA groups, except normal saturated fatty acids, which showed a strong negative association (Figure 3, Chapter 4). A relative increase in normal saturated fatty

acids has been shown to correlate with decreased diversity (Table 7, Chapter 2). Thus, a low level of normal saturated fatty acids at the sites with high organic carbon content suggests increased diversity and possibly higher resilience at these sites. Only the normal saturated fatty acids showed a positive association to the percentage ground and crown vegetation cover, with all other major PLFA showing different degrees of negative association. The Ogies Reference site was the only reference site with a high percentage ground and crown vegetation cover and high normal saturated fatty acids. This site had the lowest concentrations of all other major PLFA groups. The Newcastle reference site showed the highest biomass and occurrence of polyunsaturated fatty acids, indicative of fungi, even though it had low percentage vegetation cover. The Ermelo reference site, which also had a relatively low percentage vegetation cover, showed a high amount or terminally branched saturated and monounsaturated fatty acids, indicative of Gram-positive and Gram-negative bacteria, respectively (Figure 2, Figure 6, Chapter 4).

From the results obtained in this study it is evident that the organic carbon content of soil has a very important influence on the soil biochemical and microbiological properties and is paramount in the maintenance of good soil quality. Although microorganisms represent only a fraction (1-8%) of the total SOM, they affect vegetation growth by acting as catalysts for biotransformations (Souza Andrade et al., 2003). The recycling of organic compounds by the microbial population also enhances processes of decompostion, mineralisation and adsorption of nutrients by plants. In turn, this contributes to plant productivity and resistance to plant diseases. Other advantages of improved levels of organic carbon in soil pertain to the physical and chemical characteristics of the soil. High organic carbon content favours cation exchange capacity (CEC); aggregate stability and soil structure; water-holding capacity; root penetration and adsorption of microorganisms, nutrients and elements such as manganese (Díaz-Zorita and Grove, 2002; Souza Andrade et al., 2003). All of these factors in turn contribute to conditions that further favour microbial activity and nutrient cycling, making the aim of achieving sustainable ecosystems more viable. Results obtained during this study indicate that it is possible to improve several soil properties during rehabilitation processes and to get closer to the aim of establishing self-regulating ecosystems with associated self-sustainable vegetation growth. However, it is in

all probability impossible to rehabilitate a disturbed environment to the same standard of soil quality that existed before perturbation. The physical, chemical and biological changes that take place in the soil environment during excavation for use as topsoil cover are simply too far-reaching to negate on a human time-scale.

3. GENERAL CONCLUSIONS

The relevance of the results obtained in this study must be understood in the context of a search for a quality index as an indicator of sustainable management for the successful rehabilitation and revegetation of coal discard sites. Results obtained from the multifactorial analysis of microbiological and biochemical properties of soil illustrate that these properties are all interrelated and extensively influenced by the physical and chemical characteristics of soil being studied. It is evident that the absence or low percentage of plant cover and associated lower levels of organic matter content of the topsoil covers have an adverse effect on enzymatic activities as well as microbial population size. The lower microbial activity in the soils with lower plant cover appear to result in lower soil quality and fertility which could have a significant effect on the sustainable revegetation of the topsoils used as cover on the coal discard sites. Based on the results obtained during this study, it is evident that the characterisation of microbial enzymatic activities and microbial community structure could serve as rapid assessment criteria for the evaluation of successful revegetation and subsequent rehabilitation of sites associated with discard mining material.

In this study a positive association was observed between the percentage carbon, microbial activity and microbial biomass. The possibility exists that this association could be attributed to unsatisfactory levels of organic carbon in the topsoil obtained from the seven coal discard sites, suggesting that organic carbon is the limiting substrate for microbial activity in these soils. Furthermore, this could imply that it would only be possible to make a definite prediction about the association between vegetation cover and organic carbon content once it is established that organic carbon is indeed not the limiting factor for microbial growth. In order to do this, it would be necessary to determine specific effects of

additions of inorganic nitrogen and phosphorus on the microbial communities in the topsoil layers and whether these elements are also limiting to microbial growth.

According to Doran (2002), "the multifaceted and changing nature of sustainability is difficult to define but is aptly captured by ... 'an agriculture that sustains the people and preserves the land'". The setting and monitoring of soil quality indicators is of great importance to ensure that soil function is maintained in current land use as well as for potential future uses. It is of the essence, specifically seen in the light of increasing demands being placed on natural habitats to support anthropogenic activities, to reassess and redirect research and technology objectives in order to improve management practices and help ensure sustainable utilisation of soil resources. If a concerted effort is made at all levels to address the problems associated with the rehabilitation of degraded soil ecosystems, it will hold this environmental, social and economic benefits for South Africa and countries with similar dilemmas (DEAT, 1999). However, there are some there are key issues, concerning both conceptual and practical applications, that need to be addressed, and significant knowledge gaps need to be further researched before a soil quality index could be established (Pankhurst et al., 1997). Some of these issues include the following:

- Ongoing difficulty to define soil quality;
- The absence of clear base-line data to act as a reference point for soil quality assessments;
- Identification and standardisation of suitable assays and data interpretation that represent the summed response of the soil ecosystem;
- How to deal with systems that exhibit inconsistent responses to perturbations;
- The need for complex methodologies and technical expertise;
- High levels of temporal and spatial heterogeneity that affect all measurements in most systems;
- Lack of validation of biological indicators in diverse situations (e.g. soil types and climatic zones);
- The requirements for estimates of soil quality may vary between end-users (e.g. farmers and researchers).

(Pankhurst et al., 1997)

The combination of chemical-ameliorative and ecological approaches is applied with relative success in rehabilitation efforts today, but it is necessary for a more holistic approach towards ecological restoration to be phased in to ensure ecological sufficiency of the restored vegetation cover (Van Wyk, 2002). The science and technology of ecological restoration must be developed within a sustainability framework (Cairns, 2000) to be a successful long-term solution.

4. RECOMMENDATIONS AND FUTURE RESEARCH

Based on the results obtained and conclusions drawn during this study and from related research by other authors, some approaches are proposed for consideration with regard to the management strategy of the coal discard sites managed by Ingwe mines, and possibly the rehabilitation of other similar sites.

It would be beneficial to ensure that levels of organic carbon in topsoil layers are improved and maintained at a sufficiently high level until the system becomes ecologically self-sustainable. The importance and advantages of a high organic carbon content to soil quality has already been discussed and is obviously vital in achieving a stable ecosystem with established nutrient cycles which could result in the establishment of self-sustaining vegetation cover. Past management strategies involved the addition of organic matter to the topsoil layers of the coal discard sites in the form of kraal-manure and the regular removal of biomass (cutting of grass). At Ingwe mines the biomass has to be removed before it reaches excessively high levels that would result in the collapse of grasses and dying off of biomass. It has been shown that the application of dairy-feedlot manure significantly increases carbon, nitrogen and soil microbial biomass (Peacock et al., 2001). However, the problem with the current management strategy is that self-sustaining nutrient cycles cannot be established because the aboveground biomass and associated nutrients are continuously removed. This requires the continuous exogenous addition of fertilisers and organic matter, making the practice unsustainable and financially unfavourable. A possible solution would be to employ an intensive grazing management system. Electric fencing should be used to systematically move cattle along the site, allowing them to graze until the desired volume

of biomass is removed before moving them to the next area. At the same time, organic matter would be provided to the system in the form of manure during wastage. Although cattle-manure has a high ammonium content the risk of acidification of the soil environment is small because ammonium is volatile and should therefore only remain in the manure for the short-term. Another important aspect that should be taken into account is that it would be beneficial to limit the amount of nitrogen added to the soil environment to control the biomass yield. The reason being that high biomass yields would deplete the nutrients in the soil environment; also the aim should be to establish a sustainable ecosystem, not artificial pasture. Furthermore, nitrogen should always be added in the form of nitrate to coal discard sites. This would promote the buffering capacity of the soil because it results in the alkalinisation of the topsoil layers.

Another important management strategy would be to prevent, as far as possible, the occurrence of fires. Fires can be extremely destructive to vegetation cover and subsequently interfere with normal ecosystem functioning, such as the maintenance of water supplies, protection against erosion, and the accumulation of nutrients (Saá et al., 1996). During fires organic matter and phosphorus are lost from the system; the latter becoming mobilised. The decrease in the content of residual phosphorus translates into a loss to the ecosystem of a reservoir of phosphorus for plant nutrition. These losses negatively affect the establishment of nutrient cycles (Hungerford et al., 1991) and make it once more necessary to add large amounts of phosphorus fertiliser and organic matter to the soil. Other negative effects of fire include the following: denaturing or inactivation of enzymes crucial to ecosystem processes (Saá et al., 1996); loss of stored water and amino acids; volatilisation of nitrogen and sulphur; reduced soil porosity, water movement and water holding capacity; and death of plant tissue and seeds. Microorganisms are affected directly by heat and indirectly by physical and chemical changes in soil (Hungerford et al., 1991).

Temporal and spatial monitoring of rehabilitation sites to establish long-term trends concerning the relationship between microbial community function and structure, physical and chemical characteristics and the establishment of self-sustaining vegetation cover

would be valuable, as would the analysis of other rehabilitation sites. The latter could be used to establish whether results obtained could be related to other rehabiliation sites of different discard materials.

Another practical consideration would be to evaluate the difference in soil microbial community function and structure in topsoil excavated in the traditional manner (all soil horizons are mixed) and excavation while maintaining the intactness of the soil horizons. In the latter approach it is suggested that soil horizons are excavated separately and replaced in reverse order, thus maintaining the integrity of the physical, chemical and biological properties of the soil. It would also be useful to evaluate the effect of storage of excavated topsoil on the physical, chemical and biological characteristics of soil quality including the analysis of microbial community function and strucure.

Future research in this and similar areas could consider a number of alternatives and/or additional options in terms of analyses to assess the quality of the soil environment.

When establishing fungal/bacterial ratios it could be more significant to determine soil fungal biomass by quantifying ergosterol, as opposed to using only the quantity of 18:2ω6. Measurement of soil ergosterol concentration has been shown to be a useful estimate content of the living soil fungal biomass (Montgomery et al., 2000).

The total organic carbon content of any soil includes both organic carbon and microbial carbon. The latter constitutes a small fraction of the total soil carbon but is an important reservoir of nutrients for plants (Souza Andrade et al., 2003). Quantification of soil microbial biomass carbon (by for example, chloroform fumigation-extraction) (Hargreaves et al., 2003) and the determination of the ratio of microbial carbon ($C_{microbial}$) to organic carbon ($C_{organic}$), could be valuable since it has been shown that the $C_{microbial}$:$C_{organic}$ ratio is affected by management practices (Souza Andrade et al., 2003). The labile nature of microbial carbon implies high variability in both spatial and temporal trends and it would therefore be most useful as an assessment tool of long-term trends (Souza Andrade et al., 2003; Hargreaves et al., 2003). The advantage of assessing soil microbial carbon is that it

changes faster than soil organic carbon or biomass and gives a better indication of the direction of such change (Hargreaves et al., 2003). Furthermore, a distinction should be made between viable and non-viable microbial biomass. Phospholipid fatty acid (PLFA) analysis provides a measure of viable microbial biomass, while non-viable microbial biomass could be determined by the chloroform fumigation-extraction (CFE) or chloroform fumigation-incubation (CFI) methods (Bailey et al., 2002). The diglyceride fatty acid (DGFA) to PLFA ratio could also provide an estimate of the ratio of non-viable microbial to viable microbial biomass (Dowling et al., 1986).

Another potential assessment tool is the use of radiolabelling. Carbon in selected vegetation species could then be tracked through the system until it can be detected in microorganisms. The labelled carbon could be tracked in two ways: through the ecosystem when the vegetation is consumed by cattle and when it is not. This would present an estimate of the time it takes to complete the nutrient cycling process and which of the two cycles is the least time consuming. Subsequently, it would be possible to predict how long the rehabilitation process would take to reach a sustainable level and to determine what fractions of the carbon are transformed into organic matter or taken up by microorganisms.

The determination of respiration rates (substrate induced respiration, basal respiration) as a measure of microbial activity should be carried out in conjunction with assays of enzymatic activity to determine if both assessments provide equally reliable results.

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